Food Research

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The Vital Story of
RICE ENRICHMENT

by Science Writer

The story of rice enrichment begins in the Philippines in 1910. In that year and place, Dr. R. R. Williams encountered the clue that started him on the road to the first successful synthesis of thiamine (vitamin B_1) in 1936. It was the curative effect against beriberi of an extract of rice polishings.

The problem of beriberi, while not serious in the United States, is recognized as severe, with high mortality, in the Philippines and other Asiatic countries where the population exists principally on white rice.

It is known that beriberi is caused by dietary deficiency and that factors in those parts of the rice which are removed in the milling are effective in combating the disease.

If rice could be milled to the whiteness desired by rice eaters and, at the same time, be made to contain the anti-beriberi factor, a giant stride could be taken to improve the health of rice-eaters. The use of white rice by these peoples, coupled with illness and death from beriberi, poses a problem for public health officials.

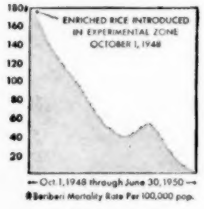
The answer to this problem was found in the laboratories of Hoffmann-La Roche. The researchers discovered a process by which white rice could be fortified. The method employs coatings of thiamine, niacin, and iron on rice grains and a final, outer coating of edible material to protect the vitamins against deterioration and pre-liminary washing and rinsing. For economy, large amounts of vitamins are applied to small amounts of rice to make a "premix." One pound of this enrichment premix is then mixed with each 199 lbs. of ordinary milled white rice at the rice mill. Each 8 ounces of this nutritionally-improved rice provide 1 mg. of thiamine, 8 mg. of niacin and 6.5 mg. of iron. These levels are commonly used but higher levels are easily obtainable.

A research project was established to study the effect on health of feeding white rice so enriched to a *population* in the Far East. Through the cooperation of the Government of the Philippines, the U. S. Public Health Service, the Williams-Waterman Fund, the Philippine National Rice and Corn Corporation, and Hoffmann-La Roche, a controlled experiment on the Bataan peninsula was set up. Medical examinations, under the direction of Dr. Juan Salcedo, now Secretary of Health for the Philippine Republic, were made of more than 12,000 people in the area before the enrichment of rice began. Nearly 13% had beriberi but most were symptom free a year later.

Enriched white rice was put into commerce in the experimental area, after full publicity about the program, on October 1, 1948. Careful monthly records were kept of the mortality from beriberi in the experimental and control areas.

The results of the test proved overwhelmingly the value of enriched white rice.

At the end of 2 years, with over 90,000 persons under this great nutritional test, the beriberi mortality rate per 100,000 population had dropped from approximately 190 in the experimental area to zero! And, from the evidence of the medical examinations, it was found that almost 90% of those who had displayed beriberi symptoms earlier were now free of them or greatly improved. Literally thousands of people were helped to better health.



The vital story of the Bataan experiment shows once again the value of enrichment to *entire peoples.* The moral is obvious: when good foods are made better, everyone benefits.

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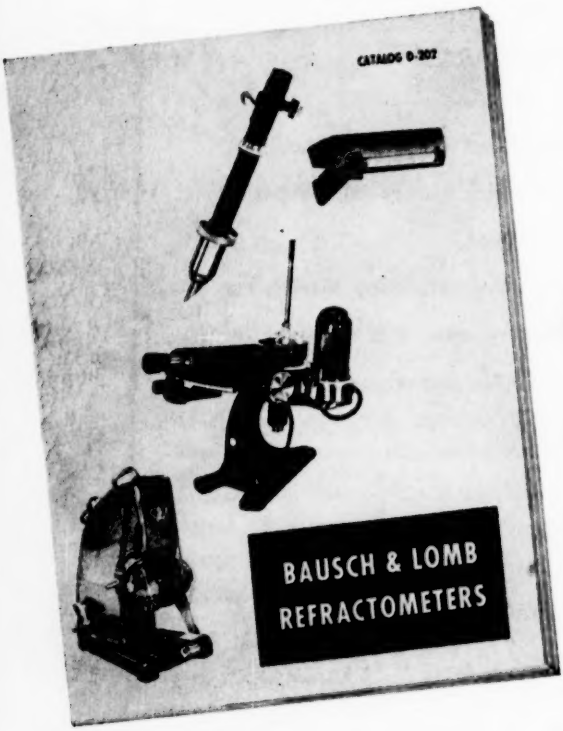

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PECTOLYTIC ENZYMES OF GARLIC[a,b]

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(Manuscript received June 12, 1952)

Wenzel and Fabian *(8)* in 1945 showed that garlic could cause softening of pickles. They found the rate of softening greatest where either a large amount of garlic or moldy garlic was added to the pickles; the rate of softening was least or nil where dehydrated or sterilized garlic was added. With these findings in mind, it was decided to investigate the pectolytic enzyme content of fresh garlic, dehydrated garlic, and moldy garlic and to determine some of the properties of these enzymes.

EXPERIMENTAL METHODS AND RESULTS

Determination of pectinesterase in fresh and dehydrated garlic. The pectinesterase content of garlic was determined by measuring the increase in free carboxyl groups to pectin. The procedure followed closely that of Hills and Mottern *(4)* and Bell, Etchells, and Jones *(3)*.

The garlic extract used in these experiments was prepared by breaking clusters of garlic into individual cloves which were examined very carefully for any signs of microbial decomposition. Only those cloves that were sound and normal in appearance were used. They were placed in a quart mason jar and held in a deep freeze unit at a temperature of −20°C. When ready to use, the cloves were allowed to thaw partially and because of the low moisture content of garlic (64-66%) enough distilled water was added to the cloves to secure a consistency necessary for proper maceration of the garlic tissue. Approximately 500 g. of garlic cloves were macerated in 270 ml. of distilled water in a Waring blendor for 20 minutes. Enough NaCl was added to give a 2.4% concentration to the macerate. The pH of the garlic-distilled water macerate was 5.6.

According to Kertesz *(6)* pectinesterase can be easily desorbed from water insoluble cellular tissues by the use of fairly strong salt solutions; by raising the pH of the tissue macerate above 5; or by using a combination of these two conditions.

After thoroughly blending, the macerate was squeezed by hand through several layers of cheesecloth and then filtered through coarse filter paper using a Büchner filter flask. The extract was layered with toluene and stored at 7°C. until used.

The quantitative test for pectinesterase in garlic was made by adding 200 ml. of a 1% pectin solution,[c] 5 ml. of 0.2 M sodium oxalate and sufficient 2 M NaCl to give a final concentration of 0.15 M NaCl in the reaction mixture after which sufficient distilled water was added to make the final volume 500 ml. This mixture was incubated at 30°C. and 25, 40, and 50 ml., respectively, of the garlic extract added to it and the pH adjusted to 7.5 with NaOH and maintained at this pH throughout the experiment. Fig. 1 shows the de-esterification curves obtained for the different quantities of extract used.

It can be seen that with an increase in time, the activity of the enzyme continued until a limiting value was reached. It may also be noted that the limiting value was reached quickest where the greatest amount of garlic extract was employed and that the limiting values were the same for all the extracts tested.

---

[a]Presented at the Twelfth Annual Meeting of the IFT at Grand Rapids, Michigan, June 10, 1952.

[b]Journal article No. 1413, Michigan Agricultural Experiment Station.

[c]Pectin was obtained from California Fruit Growers Exchange and was labeled "Special Pectin for Enzyme Testing No. 447 U-7."

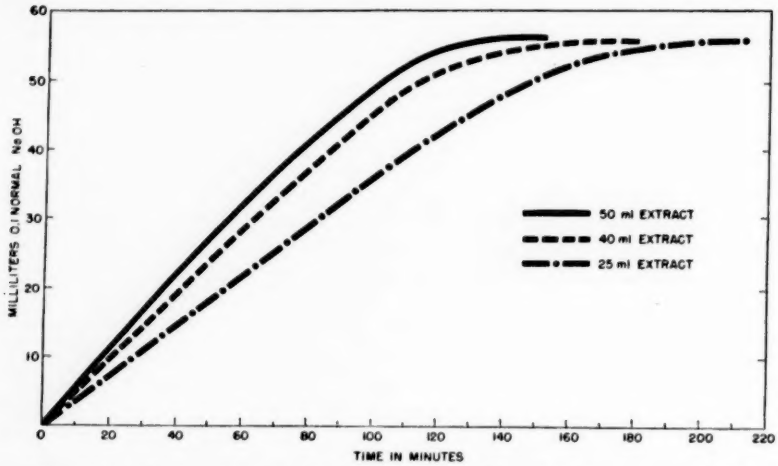

Figure 1. Time curves for the de-esterification of pectin by 25, 40 and 50 ml. quantities of garlic extract.

The results may also be expressed in pectinesterase (PE) units. This unit is defined by Bell *et al. (2)* as the number of milliequivalents of ester bonds hydrolyzed per unit volume or weight of enzyme material at pH 7.5, at 30°C., and with 0.15 M NaCl when acting on a 0.4% pectin solution. The PE unit for the fresh garlic ranged from 0.156×10^{-2} to 0.114×10^{-2} per ml. of extract.

Commercial dehydrated powdered garlic and dehydrated garlic flakes were also tested for the presence of pectinesterase. The method used was similar to that described previously. The PE units obtained for these dehydrated garlic products were about one-fourth less than the PE units obtained for fresh garlic on a dry weight basis.

Effect of salt on garlic pectinesterase. The effect of salt on garlic pectinesterase was studied. Salt concentrations of 0.88%, 3.0%, and 3.7% were established in the reaction mixture. The latter two percentages of salt correspond to the salt range of Kosher dill pickles. The reaction was allowed to proceed for a 30-minute period. The amount of 0.1 N NaOH consumed in the 30-minute period was used to determine the enzymatic activity. Table 1 shows the activity found at different salt levels.

TABLE 1

Activity of garlic pectinesterase at various salt concentrations

Salt concentration	Ml. of 0.1 N NaOH used	Relative activity,
		%
0.15 M (0.88%)	6.14	100
0.50 M (3.0%)	4.41	72
0.63 M (3.7%)	3.45	56

It may be seen that as the concentration of salt was increased, the activity of the enzyme was decreased. However, pectinesterase present in garlic still had an activity of 56 to 72% in the salt concentration range of Kosher dill pickles.

Effect of pH on garlic pectinesterase. The next study was made on the effect of pH on garlic pectinesterase and pH levels of 7.5, 6.0, 5.0, and 4.0 were established in the reaction mixture. One-tenth normal HCl or 0.1 N NaOH was used to adjust the pH. This reaction was also carried out for a 30-minute period. The total amount of 0.1 N NaOH consumed in the 30-minute period was used to determine the activity of the enzyme. The results are given in Table 2.

TABLE 2

Activity of garlic pectinesterase at different pH levels

pH	Ml. of 0.1 N NaOH used	Relative activity,
		%
7.5	9.89	122
6.0	7.22	100
5.0	3.47	48
4.0	.36	5

It may be noted that as the pH was decreased from 7.5 the activity of the garlic pectinesterase was diminished. At a pH of 4, the garlic pectinesterase activity was slight. It is appreciated that these values are relative since they would depend upon the optimum pH for this particular pectinesterase. In Table 2 it was assumed that the optimum pH was 6 and this was assigned a value of 100 and the relative values calculated from this. From pH 6.5 up, the reaction medium itself starts to hydrolize pectin which doubtless explains the high value at pH 7.5.

Effect of heat on garlic pectinesterase. The effect of heat on garlic pectinesterase was studied because of the wide use of fresh garlic cloves in making certain types of pickles such as genuine, processed, and fresh pasteurized dill pickles.

The temperature used to inactivate the enzyme was 72°C. which is equivalent to 165°F., the temperature used to pasteurize genuine, processed, and fresh dill pickles. Genuine and processed dill pickles may or may not be pasteurized depending upon the equipment available and the manufacturer, but, of course, it is necessary to pasteurize the fresh dills; otherwise they would spoil immediately.

The method was similar to that used by Kertesz in his studies on heat inactivation of tomato pectinmethoxylase (pectinesterase). The test was as follows: A 1% pectin solution was made to which was added 15 drops of methyl red indicator to every 100 ml. of pectin solution. The pH of the pectin solution was adjusted to 6.2 with 0.1 N NaOH. Twenty-five ml. of the 1% pectin solution and 5 drops of toluene were placed into 125-ml. flasks. Five ml. quantities of the garlic pectinesterase extract were placed in 20-ml. pyrex test tubes. The test tubes were placed in a rack and immersed in a 72°C. water bath to inactivate the enzyme. The tubes were allowed to remain in the bath for various lengths of time (Table 3) after which the contents of the tube were emptied in the flasks containing the 1% pectin solution, mixed thoroughly and incubated at 30°C. for 24 hours. At the end of this time the pH was taken. If the enzyme was inactivated, the pH would not be lowered and the indicator would still have its yellow appearance. If the enzyme was not inactivated the pH would be lowered with the formation of a red color due to action of the enzyme on the pectin. The results of this experiment are given in Table 3.

TABLE 3

Effect of heat on garlic pectinesterase

Time in water bath	Temperature of tubes in bath, °C.	Activity	pH of pectin after 24 hours
30 sec.	45	+	3.95
45 sec.	54	+	3.97
1 min.	60	+	4.00
2 min.	70	+	4.55
3 min.	72	+	5.72
4 min.	72	+	5.62
5 min.	72	—	6.31
7 min.	72	—	6.32
10 min.	72	—	6.40
12 min.	72	—	6.40

Since 5 ml. of the garlic pectinesterase was used, it was not possible to heat this quantity to 72°C. immediately. A thermometer suspended a short distance from the bottom of the tubes gave the temperature of the extract in the tubes. The enzyme was not inactivated until a temperature at 72°C. had been reached at the end of 5 minutes. This indicates that 165°F.(72°C.) for 15 min., which is the time and temperature used to pasteurize pickles, is ample to inactivate pectinesterase since it was inactivated under the conditions of the experiment in 5 min. at 72°C.

POLYGALACTURONASE-LIKE ENZYMES IN FRESH GARLIC, DEHYDRATED GARLIC, AND MOLDY GARLIC

Although polygalacturonase is generally not found in higher plants, several workers have shown that pectolytic enzymes similar to polygalacturonase exist in higher plants. Bell *(1)* has demonstrated a polygalacturonase-like enzyme in cucumbers. McColloch and Kertesz *(7)* reported this enzyme to be present in ripe tomatoes. Because of these findings it was decided to test garlic and dehydrated garlic products for this enzyme.

The method used was similar to that of Bell *et al. (3)*. Briefly, it consists of measuring the loss in viscosity of a 3% pectin solution caused by the enzyme preparation. Garlic extracts of fresh and dehydrated garlic were prepared as previously described and placed in the 3% pectin solution which was prepared in phthallate buffer at pH 4. After 1 and 7 days of incubation the mixtures were allowed to drop through a 25-ml. volumetric pipette. The dropping times were recorded and the percentage loss in viscosity was calculated by comparing the dropping times of the active extracts to those of extracts that had been inactivated by heat.

No pectolytic enzyme could be demonstrated by this method of testing in the garlic extracts. It was therefore concluded that sound garlic was probably free of polygalacturonase-like enzymes. It was then decided to look for this type of enzymatic activity in decomposed garlic.

The literature cites many fungi capable of producing pectolytic enzymes. Certain genera, such as *Penicillium* and *Aspergillus*, are commercially used to produce pectolytic enzymes for clarifying fruit juices.

Wenzel and Fabian *(8)* found *Aspergillus* and *Penicillium* molds on garlic and attributed the formation of pectolytic enzymes by these fungi as the cause of spoilage in genuine Kosher dill pickles.

Two molds were isolated from decomposed garlic. These were identified as *Penicillium canescens* and *Fusarium oxysporum*. When these molds were grown on the pectin medium of Manchester and Baier it was found that *P. canescens* liquefied the pectin readily while *F. oxysporum* liquefied it slowly and only partially.

These molds were then grown in pure culture on sterile garlic cloves. Another set of cloves was run as an uninoculated control. After 3 weeks of incubation, the inoculated cloves were completely covered with growth and very badly decomposed, while the uninoculated control showed no change.

Extracts were prepared from, a, *P. canescens*, b, *F. oxysporum*, c, the decomposed, moldy garlic, d, from garlic from the uninoculated control, and e, from garlic that had been allowed to decompose naturally. The method of preparation of these extracts has already been described. A known source of pectolytic enzyme, "Pectinol M," in a concentration of 0.1% was also tested.

The extracts were added to 3% pectin solutions and the dropping times were taken at various intervals. At the end of 6 days, the extracts from naturally decomposed garlic and from *P. canescens* decomposed garlic showed a loss in viscosity comparable to the 0.1% "Pectinol M" which was about 80%. However, the *F. oxysporum* decomposed garlic extract showed no loss in viscosity. This would indicate that not all molds are capable of producing a polygalacturonase-like enzyme. This doubtless is the exception rather than the rule, however, since all samples of naturally decomposed garlic tested and *P. canescens* did produce this enzyme.

SUMMARY

Under the condition of these experiments the results may be summarized as follows:

Garlic was found to contain the enzyme pectinesterase in amounts of 0.156×10^{-2} to 0.114×10^{-2} PE units per ml. depending on the amount of extract used.

Garlic pectinesterase showed decreased activity at 3.0 and 3.7% NaCl and at pH 4 which is the amount of salt and acid commonly found in dill pickles.

Pectinesterase was found in powdered garlic and garlic flakes but showed only approximately one-fourth of the activity of the fresh garlic buds.

Pectinesterase was inactivated in 5 minutes at 72°C. The pasteurization time recommended for pickles is 72°C.(165°F.) for 15 minutes.

Polygalacturonase-like enzymes could not be demonstrated in either fresh or dehydrated garlic products.

A polygalacturonase-like enzyme was found in all samples of naturally decomposed garlic buds and in garlic decomposed by *P. canescens* but not garlic decomposed in *F. oxysporum* which indicates that not all molds which decompose garlic produce this enzyme.

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SURVIVAL OF STAPHYLOCOCCI AND SALMONELLAE EXPERIMENTALLY INOCULATED INTO SALAD DRESSING PREPARED WITH DRIED EGGS

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(Manuscript received May 19, 1952)

A greatly improved dried egg product *(1)* is on the market today, but limitations are still placed on the use of dried eggs in food cookery. The U. S. Dept. Agr. bulletin 136, 1950 edition, *(7)* advises against using dried eggs in many dishes, including scrambled eggs, puddings, and cooked salad dressings. Goddard *(2)* ascribes this extensive limitation to the fact that species of Salmonella have been found living in dried eggs. Evidently the temperature used in preparing these dishes is not considered sufficient to destroy pathogenic organisms.

Johns and Berard *(4)* state that the occurrence of practically pure cultures of bacteria in dried eggs suggests that a single egg is responsible for the contamination. Solowey *(8)* reports that since the shell egg is an individual package, contamination is not so widespread as in bulk liquid, frozen or dried eggs. However, with modern methods of dried egg production, including pasteurization and acidification before drying, there is less danger of survival of contaminating organisms in dried eggs *(3, 6)*. Solowey, McFarlane, Spaulding, and Chemerda *(10)* report that although Salmonella organisms in food products constitute a potential hazard, the contaminated egg powder has rarely been responsible for Salmonella enteric infections.

Most of the bacteriological reports in the literature are on dried eggs themselves and not on foods prepared with them. McCullough and Eisele *(5)* report that tests on the pathogenicity of 2 strains of Salmonella isolated from dried eggs have proved very toxic to humans. The heat necessary to cook dried eggs is not always sufficient to destroy Salmonella. Solowey and Calesnick *(9)* report that test scrambled eggs prepared from artificially or naturally contaminated reconstituted dried eggs frequently yield viable Salmonella. They suggest the holding time of reconstituted dried egg should never exceed 4 hours. Some of the puddings prepared in our laboratory (unpublished data) with experimentally inoculated dried eggs also contained Salmonella. However, heating in the presence of an acid, as is done in preparation of a cooked salad dressing, should destroy pathogenic organisms found in foods. Wethington and Fabian *(11)*, in a study on experimentally inoculated commercial and laboratory prepared fresh and frozen egg mayonnaise and salad dressing with Staphylococci and Salmonellae, considered mayonnaise and salad dressing, due to their acid content, not a probable source of food poisoning. Their experiments do not state, however, under what conditions the salad dressing and mayonnaise were prepared.

This study was made to establish conditions of temperature and acidity necessary for preparing a salad dressing that is safe from either contamination by the dried eggs themselves or by the food handlers.

EXPERIMENTAL

A basic formula for cooked salad dressing typical in flavor and texture was developed using dried eggs (Table 1). The eggs were reconstituted using 12 g. with 30 ml. of water as equivalent to one fresh egg. The holding time for the reconstituted egg was never over 15 minutes. One-half minute was considered necessary for cooking the egg and the temperature of the mixture thus obtained was from 84° to 86°C. The sugar and vinegar were increased to produce variation I, II, III, and IV (Table 1). The pH was determined electrometrically on the Beckman pH meter at 25°C.

TABLE 1

Basic formula and variations

	Fat	Flour	Milk	Water	Dried egg	Sugar	Salt	Dry mustard	Paprika	Vinegar	pH as tested
	g.	*g.*	*ml.*	*ml.*	*g.*	*g.*	*g.*	*g.*	*g.*	*ml.*	
Basic Formula	26	18	122	150	12	28	3.8	2	.5	42	4.56
Variation I	26	18	122	150	12	48	3.8	2	.5	70	4.21
Variation II	26	18	122	150	12	72	3.8	2	.5	91	4.08
Variation III	26	18	122	150	12	96	3.8	2	.5	126	3.95
Variation IV	26	18	122	150	12	120	3.8	2	.5	252	3.40

The bacteria used in this study were obtained from cultures from the Bacteriology departments of the University of Missouri Medical and Veterinary Medical Schools. A food poisoning type of Staphylococcus, *Micrococcus pyogenes* var. *aureus (Staphylococcus aureus)*, and 6 species of Salmonella, *Salmonella typhimurium, Salmonella enteritidis, Salmonella pullorum* (19), *Salmonella pullorum* (20), *Salmonella gallinarum, Salmonella choleraesuis,* were used in this study.

Five culture checks were made to test for possible bacteria in the dried eggs. Twelve grams of dried egg were mixed with 30 ml. of water for one minute under sterile conditions. The reconstituted egg was transferred immediately and after 1, 2, 3, 4, and 24 hours to Bacto-Tryptose agar (Difco) and SS (Shigella-Salmonella) agar (Difco) plates to check for pathogenic organisms. The plates were incubated for 24 hours at 37°C. and the growth on the plates was identified by morphologic and growth characteristics. No pathogenic bacteria were found in these few culture checks.

pH study. The basic salad dressing formula and four variations (Table 1) using different proportions of sugar and vinegar were used in this study. Thirty grams of each of these 5 salad dressings were put into sterilized flasks. One milliliter of a 24-hour nutrient broth-bacteria culture was added to each and thoroughly mixed by shaking for one-half minute. The contaminated salad dressing was held at room temperature and transfers were made immediately and after 1, 2, 3, 4, and 24 hours. The Staphylococcus was streaked onto tryptose agar plates to check for growth. The Salmonella contaminated samples were streaked onto the selective SS agar plates to check for possible growth. All plates were incubated at 37°C. for 24 hours. Any plates with scanty growth were incubated an additional 24 hours to check for slow growing strains of bacteria. If bacterial colonies were present, they were identified by growth characteristics. If growth occurred on the SS plates, representative colonies were transferred to Kliger iron agar (Difco) slants to prove the identity of Salmonella organisms. These slants were incubated at 37°C. for 24 hours and compared with checks made on Kliger iron agar slants inoculated with a drop of the original broth-bacteria culture.

Temperature study. The 7 organisms listed above were used in the basic formula in this part of the study. The reconstituted egg mixture was contaminated by using

TABLE 2

Viability of bacteria in cooked salad dressing of varying pH

Test organism	Hours held	Plate growth at indicated pH				
		4.56 Basic formula	4.21 Variation I	4.08 Variation II	3.95 Variation III	3.40 Variation IV
Staphylococcus aureus	0	+	+	+	+	+
	1	+	+	+	+	+
	2	+	+	+	+	+
	3	+	+	+	+	+
	4	+	+	+	+	+
	24	+	+	+	+	—
Salmonella typhimurium	0	+	+	+	+	+
	1	+	+	+	+	+
	2	+	+	+	+	—
	3	+	+	+	+	—
	4	+	+	+	+	—
	24	+	+	+	+	—
Salmonella enteritidis[a]	0	+	+	+	+	+
	1	+	+	—	+	—
	2	+	+	—	—	—
	3	+	+	—	—	—
	4	+	+	—	—	—
	24	+	+	—	—	—
Salmonella pullorum(19)	0	+	+	+	—	+
	1	+	+	+	—	—
	2	+	+	—	—	—
	3	+	+	—	—	—
	4	+	+	—	—	—
	24	+	—	—	—	—
Salmonella gallinarum	0	+	+	—	—	—
	1	+	+	—	—	—
	2	+	+	—	—	—
	3	+	+	—	—	—
	4	+	+	—	—	—
	24	+	—	—	—	—
Salmonella pullorum(20)	0	+	—	—	—	—
	1	+	—	—	—	—
	2	—	—	—	—	—
	3	—	—	—	—	—
	4	—	—	—	—	—
	24	—	—	—	—	—
Salmonella choleraesuis	0	+	—	—	—	—
	1	—	—	—	—	—
	2	—	—	—	—	—
	3	—	—	—	—	—
	4	—	—	—	—	—
	24	—	—	—	—	—
Control	0	—	—	—	—	—
	1	—	—	—	—	—
	2	—	—	—	—	—
	3	—	—	—	—	—
	4	—	—	—	—	—
	24	—	—	—	—	—

[a]A slow growing strain.

10 ml. of inoculated broth in place of part of the water. Special emphasis was placed on keeping cooking temperature constant for each bacteriological test. The white sauce was cooked for one minute at 98°C. The contaminated egg mixture was added and cooked for one-half minute to a temperature of 84° to 86°C. After the salad dressing was made, the same conditions and procedures were used to check bacterial growth as in the pH study.

RESULTS AND DISCUSSION

Influence of pH. Table 2 shows a complete record of the growth of bacteria at the 5 pH's studied. *S. cholaraesuis*, *S. pullorum* (20), *S. gallinarum*, and *S. pullorum* (19), respectively, were inhibited in a salad dressing of a pH of 4.21 or higher. The more resistant of the Salmonella to the acid condition of the salad dressing were *S. enteritidis* and *S. typhimurium*. The growth of these microorganisms was inhibited at a pH of 3.40. The most persistent of all bacteria studied was the *Staphylococcus aureus*. This organism remained viable when incubated for 4 hours in a salad dressing with a pH of 3.40. Wethington and Fabian *(11)* reported that the strains of Staphylococci they tested were more resistant to the acid conditions than the Salmonellae. The pH of the basic salad dressing considered best in texture and flavor was not acid enough to destroy all the bacteria inoculated into it. Variations I, II, and III, with progressively lower pH's, showed increasing tendency to prevent bacterial growth. Variation IV, though not desirable in texture, inhibited the growth of all bacteria tested.

Influence of temperature. The plates inoculated at each of the 5 time intervals were negative for the 7 bacteria tested. The temperature of 84° to 86°C., and the pH as obtained in the basic salad dressing formula, effectively inhibited the growth of pathogenic organisms.

CONCLUSIONS

Not all Salmonellae and Staphylococci inoculated into cooked salad dressing made with dried eggs are destroyed at the pH of the basic formula, or any palatable variation, but these pH's do not provide a favorable medium for the growth of pathogenic organisms.

Cooking dried egg in salad dressing for one-half minute or to a temperature of 84° to 86°C. destroys food poisoning organisms which are likely to be found in dried eggs or introduced into them by food handlers.

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EFFECT OF METAL IONS ON THE FORMATION OF NITRIC OXIDE HEMOGLOBIN[a]

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Development of pink color in cured meats depends on the formation of nitric oxide hemoglobin *(6)*. This process is accelerated by the use of ascorbic acid, which reduces methemoglobin to hemoglobin *(2, 7)*. Ascorbic acid also retards the loss of color since nitric oxide hemoglobin can be oxidized to methemoglobin.

A series of experiments on the development of color by nitrite and ascorbic acid *(7)* suggested the following mechanisms:

1. Oxyhemoglobin is converted to methemoglobin by nitrite.
2. Methemoglobin is reduced to hemoglobin by ascorbic acid.
3. Nitrite is reduced to nitric oxide which combines with hemoglobin.

In his original observations on the reduction of methemoglobin by ascorbic acid, Gibson *(2)* reported that the reaction is catalyzed by small amounts of iron and copper. It appeared possible, therefore, that these metals and perhaps others might play a part in nitric oxide hemoglobin formation by ascorbic acid. The present paper reports several experiments designed to test this hypothesis.

MATERIALS AND METHODS

Hemoglobin solutions were prepared by washing red cells from defibrinated beef blood with isotonic sodium chloride. The cells were laked with four parts of distilled water and stored at −20°C. after adjusting to pH 5.8 with HCl. The cellular material was filtered off before use and the stock solution further diluted to a final hemoglobin concentration in the range of 0.4% to 0.8%.

All experiments were carried out in 0.02 M phosphate buffer at pH 5.8. The concentration of nitrite was 0.02% and ascorbic acid (adjusted to pH 5.8 with NaOH before addition to the hemoglobin) 0.05% or 0.1%. After mixing, the solutions were stored directly in Evelyn colorimeter tubes in which the readings were made in order to prevent variations in oxygen tension due to pouring from one container to another.

Total hemoglobin and methemoglobin were measured by the method of Evelyn and Malloy *(1)*. Transmission at 635 mμ was used as an index of red color *(7)*. A decrease in transmission indicates oxidation of hemoglobin to methemoglobin, cholehemoglobin, or both. An increase in transmission due to reduction of methemoglobin accompanies the formation of nitric oxide hemoglobin. The absolute transmission values cannot be compared from one experiment to another, since the original hemoglobin concentrations varied.

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EXPERIMENTAL

Effect of a metal complexing agent on nitric oxide hemoglobin formation. To determine whether traces of metals normally present in the solutions used might be playing a part in the ascorbic acid catalyzed reaction, a small amount of a metal complexing agent was added to hemoglobin-nitrite-ascorbic acid solutions. The agent used was the disodium salt of ethylenediaminetetraacetic acid.* It is known to form stable, non-ionized complexes with copper, iron, and other metals *(5)*. It was added at a concentration of 0.01% to hemoglobin-nitrite-ascorbic acid mixtures held at 45° and at −20°C. Previous work *(7)*, carried out in the absence of Versene, has shown that nitric oxide hemoglobin forms readily at these 2 temperatures, although at intermediate temperatures (25° and 4°C.) the reaction is delayed and incomplete.

In the presence of the metal complexing agent, nitric oxide coupling failed to occur at 45°C. (Figure 1). Methemoglobin concentration increased in the sample containing

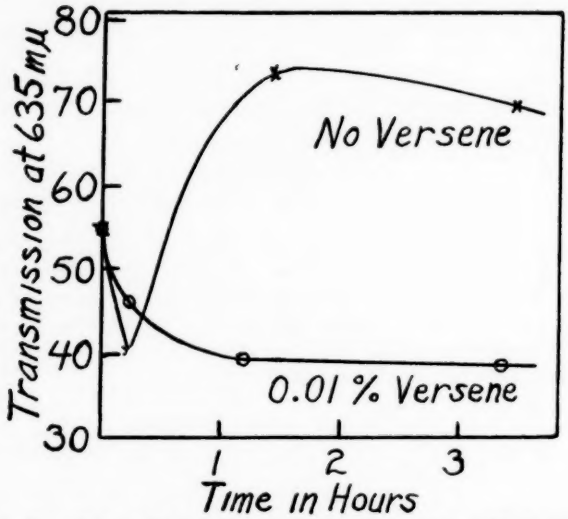

Figure 1. The effect of Versene on the formation of nitric oxide hemoglobin. All solutions contained 0.4% hemoglobin, 0.02% nitrite and 0.1% ascorbic acid in phosphate buffer, pH 5.8.

Versene from an original value of 0.069% to 0.100% at the end of one hour. In the control without Versene, the original methemoglobin disappeared completely in the same time interval. Evidently trace metals play an important role in the reaction under these conditions.

In the freezer, on the other hand, Versene had no effect on nitric oxide coupling. After storing overnight, both samples were bright red and methemoglobin had disappeared. Gibson *(2)* has suggested that 2 separate reactions may be involved in the reduction of methemoglobin by ascorbic acid, one of which may occur at small ascorbic acid concentrations, catalyzed by trace metals, and the other, more dependent on ascorbic acid concentration, taking place in the absence of metals. At the high ascorbic acid concentrations obtained on freezing, metal catalysis may not be an important factor.

The effect of added metals. Choosing a set of conditions under which the formation of nitric oxide hemoglobin is delayed and incomplete, i.e. 0.1% ascorbic acid at room temperature *(7)*, the effect of the addition of various ions was determined. Of

*This product is sold under the trade name Versene (Bersworth Chemical Co., Framingham, Mass.).

the ions, Al, NH_4, Ca, Co, Cu(ic), Fe(ous), Fe(ic), Mg, Mn(ous), Ni(ous), and Zn, all at a concentration of 5 p.p.m., Cu, Fe(ous, ic), and Zn accelerated the reaction; all others gave curves practically identical with the control. Figure 2 shows the progress of color development in the presence of cupric, ferrous, and zinc ions at concentrations of 5 p.p.m. Ferric salts were very similar to ferrous.

In order to determine a, if the metals found effective in accelerating nitric oxide hemoglobin formation might also be effective in retarding fading and b, whether their activity was specifically associated with ascorbic acid, a second experiment was performed in which the metals were added to aliquots of nitric oxide hemoglobin solutions and subsequent fading of the solutions was followed. The nitric oxide hemoglobin was

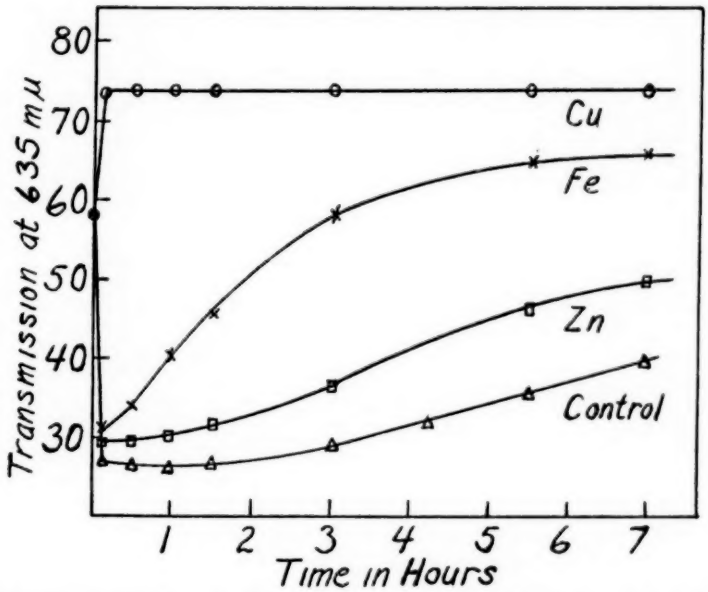

Figure 2. The effect of copper, iron and zinc on the formation of nitric oxide hemoglobin at 25°C. All solutions contained 0.4% hemoglobin, 0.02% nitrite and 0.1% ascorbic acid in phosphate buffer, pH 5.8. Ion concentration—5 ppm.

prepared in 2 ways, a, by the addition of sufficient sodium hydrosulfite to a buffered hemoglobin solution containing 0.02% nitrite to give an immediate reaction *(3)*, and b, by addition of 0.1% ascorbic acid to an aliquot of the nitrited hemoglobin solution followed by color development at 45°C. over a period of approximately 2 hours. Good color formation could be obtained with both procedures. Both reached an identical peak transmission at 635 mμ of 71. At the time of maximum color development (peak transmission), the 3 metals were added at the previous levels of 5 p.p.m. to aliquots of each solution in colorimeter tubes, and the fading of the color was followed by transmission readings (Table 1). Whereas the metals protected color in the ascorbic acid solutions, they did not in the solution reduced with hydrosulfite. In fact, copper and iron accelerated fading in the absence of ascorbic acid.

In the absence of nitrite, copper and iron, especially the former, brought about a more rapid oxidation of hemoglobin. When added alone to hemoglobin solutions, copper caused a rapid production of methemoglobin; when added with ascorbic acid, it accelerated the formation of green choleglobin. Zinc, on the other hand, slightly retarded the oxidation of hemoglobin solutions.

TABLE 1

Effect of ions on oxidation of nitric oxide hemoglobin

Ions added (5 p.p.m.)	Transmission at 635 mμ after 4 hours at 25°C. (Initial reading – 71)	
	Color developed with hydrosulfite	Color developed with ascorbic acid
Control	45	49
Copper	36	61
Iron	40	62
Zinc	47	58

As in the experiment with Versene, temperature is an important factor. Table 2 shows the effect of cupric, ferrous, and zinc ions on the formation and subsequent oxidation of nitric oxide hemoglobin at 4 different temperatures. A lower concentration of ascorbic acid, 0.05%, as well as 0.10%, was tried. Two sets of transmission figures are presented at each temperature, one taken at an early time interval during formation of nitric oxide hemoglobin, and the second at a later time, when color development had passed its peak and the solutions were fading.

TABLE 2

Effect of ions on nitric oxide hemoglobin at different ascorbic acid levels

Ion (5 p.p.m.)	Ascorbic acid	Transmission at 635 mμ (Initial value – 58)							
		−20°C.		5°C.		25°C.		45°C.	
		3 hr.	24 hr.	1 day	7 days	3 hr.	24 hr.	1 hr.	3 hr.
	percent								
Control	0.05	58	33	35	31	25	25	21	58
	0.10	69	67	43	36	32	30	29	72
Cu(ic)	0.05	59	16	70	37	73	38	69	66
	0.10	73	63	74	33	74	65	72	70
Fe(ous)	0.05	59	39	36	28	39	43	56	58
	0.10	64	61	41	31	58	59	53	57
Zn	0.05	60	33	39	39	31	35	22	40
	0.10	70	65	56	48	45	47	37	61

None of the metals had any significant beneficial effect in the freezer. With 0.1% ascorbic acid, color development was complete within a few hours without the metals. At 45°C., where color development was complete in less than 2 hours without the metals, the only advantage in their use seemed to be a more rapid original rise in transmission. Iron and zinc brought about a more rapid fading than did the control. In the refrigerator (5°C.) both zinc and copper accelerated color formation. With zinc the color took longer to develop but showed less rapid fading than copper. Iron was not effective at this temperature. The lower concentration of ascorbic acid was much less effective throughout.

Table 3 shows the effect of varying the concentration of the metallic ions, with the same concentration of ascorbic acid (0.1%). The optimum concentrations of the several metals appear to be 2 to 5 p.p.m. for copper, 5 to 10 p.p.m. for iron, and 10 to 20 p.p.m. for zinc. Of particular interest is the prolonged protective effect of zinc at the higher concentrations, especially in the refrigerator.

Methemoglobin concentrations were not followed in the preceding experiments. However, in a single experiment in which formation of nitric oxide hemoglobin was catalyzed

TABLE 3

Effect of ascorbic acid and ions at various levels of nitric oxide hemoglobin

Ion added (all solutions contained 0.1% ascorbic acid)	Ion concentration p.p.m.	Transmission at 635 mμ (Initial value – 58)							
		−20°C.		5°C.		25°C.		45°C.	
		3 hr.	24 hr.	1 day	7 days	3 hr.	24 hr.	1 hr.	3 hr.
Control	.....	69	67	46	36	34	31	28	72
Cu(ic)	1.0	70	56	65	36	69	71	73	70
	2.5	74	70	74	37	75	74	76	72
	5.0	74	63	74	33	74	65	73	68
Fe(ous)	2.5	66	48	41	36	47	62	44	65
	5.0	64	59	41	31	55	61	65	59
	10.0	60	58	42	35	62	59	66	56
Zn	5.0	73	71	58	54	53	57	45	72
	10.0	72	67	64	70	58	71	49	67
	20.0	74	69	67	73	64	72	51	57

by iron and ascorbic acid at room temperature, the iron was found to catalyze the reduction of methemoglobin by ascorbic acid. In the absence of iron, methemoglobin increased from an original value of 0.056% to approximately 0.100% and varied irregularly between 0.08% and 0.10% for the 22 hours of the experiment. In the presence of iron, after an original increase in methemoglobin immediately after the addition of nitrite *(7)* the methemoglobin decreased to zero during the following four hours, the drop paralleling closely color formation and transmission at 635 mμ, which reached a maximum in four hours. The transmission fell only slightly during the subsequent 18-hour period and methemoglobin remained at zero.

Although methemoglobin changes have not been followed closely with the other metals, at peak color development with copper and zinc, no methemoglobin was present.

DISCUSSION

It seems evident that copper, iron, and zinc can accelerate the development of nitric oxide hemoglobin in the presence of ascorbic acid and retard subsequent fading of the color. Little is known of the mechanism of the reactions involved. All 3 metals can evidently catalyze the reduction of methemoglobin by ascorbic acid under appropriate conditions (differing for the 3 metals) of temperature and concentration. Copper and iron, especially the former, also accelerate oxidation of oxyhemoglobin to methemoglobin, whereas zinc does not. Whether the metals play any part in the reduction of nitrite to nitric oxide is not known. Ascorbic acid is oxidized by nitrite *(4)*, the nitrite being presumably reduced at the same time.

Since the metals are most effective at room and refrigerator temperatures where nitric oxide hemoglobin formation is slow and incomplete in their absence, it would be of interest to test their effectiveness on color development in meats. Zinc seems of particular interest because of its prolonged protection of nitric oxide hemoglobin at refrigeration temperatures. No information is available on its activities in meats, either with or without ascorbic acid. Combinations of the metals might also prove of interest, since copper, unlike zinc, even in very small concentrations can be very effective in producing rapid initial color formation. Concentration

ranges found effective in relatively pure hemoglobin solutions may not be optimum in meat, where the metal binding activity of the meat proteins may be an important factor.

SUMMARY

Addition of a metal complexing agent, ethylenediaminetetraacetic acid, prevented formation of nitric oxide hemoglobin in hemoglobin-ascorbic acid-nitrite solutions at 45°C. Three metals, copper, iron, and zinc, were found to accelerate nitric oxide hemoglobin formation and to retard subsequent fading in such solutions under specified conditions of concentration and temperature. These metals catalyzed the reduction of methemoglobin by ascorbic acid.

Copper, in concentrations of 2-5 p.p.m., gave immediate color formation at all temperatures above freezing. Zinc (10-20 p.p.m.) was very effective in retarding fading of nitric oxide hemoglobin at refrigeration temperatures. All 3 metals accelerated color formation and retarded fading at room temperature. Neither the metal complexing agent nor added metals affected color formation in the freezer.

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NITROGENOUS CONSTITUENTS IN CITRUS FRUITS.
I. SOME FREE AMINO ACIDS IN CITRUS JUICES DETERMINED BY SMALL-SCALE FILTER-PAPER CHROMATOGRAPHY[a]

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Deterioration of processed citrus juices has been attributed to chemical changes involving amino acids *(7, 21, 30, 42)*, carbohydrates *(7, 42)*, oil constituents *(4, 23, 24, 37)*, organic acids *(7)*, polyphenols *(12)*, and compounds containing sulfur *(16, 20, 24)*. It would appear desirable to establish ultimately the identity of all constituents in citrus fruit juices, to develop methods for their estimation, and to correlate chemical changes with the loss of juice quality during processing and storage.

It is well known that amino acids and other nitrogen compounds may react with sugars, such as are present in citrus juices *(19)*, with the formation of unusual colors, flavors, and odors *(8)*. However, very little systematic work has been done to determine the character, amounts, and reactivities of the nitrogenous constituents which compose from 5 to 10% of the total solids in citrus fruit juices.

Asparagine and glutamine were detected in orange juice by Scurti and Plato *(32)* as early as 1908, and more recently in lemon juice by Solarino *(34)*. Funk *(11)* in 1913 succeeded in isolating 3 nitrogen-containing compounds, $C_9H_{18}N_2O_6$, $C_6H_7N_3O_2$, and $C_5H_{15}NO_2$, from lime juice. However, these formulae do not correspond with any known constituents in citrus juices. Yoshimura *(43)* isolated and identified betaine and stachydrin from orange juice in 1918. The last 2 compounds and putrescine were isolated from pummelo (or shaddock, *Citrus grandis*, Osbeck) by Hiwatari *(14)* in 1927. Herbst and Snell *(13)* recently isolated putrescine from orange juice. In the most recent comprehensive study, Nelson, Mottern, and Eddy *(21)* isolated arginine, aspartic acid, and choline from orange juice, in addition to asparagine and stachydrin whose presence had been reported by earlier investigators. Evidence was also presented by Nelson *et al.* for the presence of small amounts of histidine in orange juice. Swift and Veldhuis *(37)* isolated choline and a compound presumed to be ethanolamine from the lipid fraction of orange juice. Recently, cysteine and glutathione were isolated, as their S-benzyl derivatives, from orange juice by Jansen and Jang *(16)* and estimated as the free, reduced compounds in fresh and heated juices from orange, grapefruit, lemon, and lime by Miller and Rockland *(20)*.

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Newly developed small-scale filter-paper chromatography procedures have been employed in the present study for a systematic investigation of ninhydrin-reactive nitrogenous constituents in the juices of commercial and experimental hybrid varieties of citrus fruits. A preliminary report of some of this work has been published elsewhere *(30)*.

EXPERIMENTAL PROCEDURE

Preparation of samples. The citrus fruits used in the present studies are described in Table 1. Fresh fruits were hand reamed on a household-type, glass extractor and strained through a 16-mesh stainless-steel-wire screen. Commercially processed samples were used as obtained from the can. A 100-ml. aliquot of each juice was treated with 2.5 g. of Celite[c] filter-aid and filtered with suction on Schleicher and Schuell No. 589 Blue Ribbon filter paper in a 6-cm. Büchner funnel. Untreated samples of several juices gave chromatographic patterns identical with the Celite-treated juices but were more difficult to use in the chromatographic studies because of the presence of extraneous cellular material. Therefore Celite-treated juices were employed in all filter-paper-chromatography studies. Some of the filtered juices were also lyophilized and made up to exactly ⅕ of their original volume in order to minimize the amount of work necessary to prepare satisfactory chromatograms. Single-strength juices and lyophilized concentrates of the same juice samples had identical chromatographic patterns.

Determination of pH, brix, and acid. The pH of each juice was determined electrometrically with a glass electrode pH meter standardized against pH 7.00 buffer. Acidity, calculated as grams of citric acid per 100 ml. of juice, was determined by titration of 10-ml. aliquots of Celite-treated, filtered juice with 0.1655 *N* sodium hydroxide using 0.25 ml. of a 1% alcoholic solution of phenolphthalein as indicator. Brix was determined with a refractometer according to the procedure of Stevens and Baier *(35)*.

Filter paper chromatography. *General procedure.* The small-scale, two-dimensional filter-paper-chromatography technique of Rockland and Underwood *(31)* was employed for the determination of nitrogenous constituents in citrus fruit juices. The one-dimensional test-tube-chromatography procedure of Rockland and Dunn *(28)* was used for the confirmatory color tests for arginine and histidine. R_F values obtained from one-dimensional chromatograms were measured with the Partogrid *(29)*. Schleicher and Schuell No. 589 Blue Ribbon filter paper *(27)* was employed for both one- and two-dimensional chromatograms.

Single-strength or lyophilized (5:1) concentrates of the citrus juices were applied to the filter paper with micro pipettes *(28)* or an ultramicro burette *(27)*. A total of 2 microliters of single-strength citrus juice or 0.4 microliter of lyophilized concentrate was "spotted" approximately ¾ inch from one corner of the 5 x 5-inch squares of filter paper in aliquots of 1×10^{-4} ml., allowing each spot to dry completely between applications. At least 3 replicate chromatograms were prepared from each of the samples or amino acid test mixtures. Each of the replicates was chromatographed in a separate museum jar together with replicates of 2 different samples or test mixtures. Solvent travel was limited to approximately 100 mm. for both one- and two-dimensional chromatograms. The "dip" technique *(27, 31)* was employed for the application of ninhydrin reagent and development of colored areas.

Solvents. All organic solvents were fractionally distilled to remove water and trace impurities. Phenol was purified according to the procedure of Draper and Pollard *(10)*. Solvent mixtures employed for filter-paper chromatography included:

A. n-propanol : water = 70 : 30 (v/v, 25°C.).
B. phenol : water = 75 : 25 (w/v, 25°C.).
C. 2,4,6-collidine : 2,4-lutidine : water = 37.5 : 37.5 : 25 (v/v/v, 25°C.).
D. tert.-butanol : water : 85% formic acid, aq. = 69.5 : 29.5 : 1.0 (v/v/v, 24°C.).
E. phenol : water : conc. NH_4OH = 77.5 : 21.5 : 1.0 (w/v/v, 24°C.).

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[c] Mention of special instruments or materials throughout this paper does not imply that they are endorsed or recommended by the Department of Agriculture over others of a similar nature not mentioned.

TABLE 1

Description of citrus fruits studied

Sample No.	Type	Variety	Origin	Source	Condition of juice
1	Orange	Parson Brown	Florida	Local market	Fresh
2		Valencia	California	Univ. California, Los Angeles	Fresh
3		Valencia	Unknown	Commercial pack	Canned, pasteurized
4		Washington Navel	California	Local market	Fresh
5		Washington Navel	California	Commercial pack	Canned, pasteurized
6	Tangerine	Unknown	California	Local market	Fresh
7		Unknown	Unknown	Commercial pack	Canned, pasteurized
8	Grapefruit	Marsh	Arizona	Local market	Fresh
9		Marsh	California	Local market	Fresh
10		Pink	Texas	Local market	Fresh
11		Pink	Texas	Commercial pack	Canned, pasteurized
12		Unknown	Florida	Commercial pack	Canned, pasteurized
13	Lemon	Eureka	California	Local market	Fresh
14		Unknown	California	Commercial pack	Canned, pasteurized
15		Unknown	California	Commercial pack	Canned, concentrated, pasteurized
16	Lime	Unknown	Unknown	Local market	Fresh
17	Tangor	Dweet[d]	California	Univ. California, Riverside	Fresh
18	Mandarin	Kara[e]	California	Univ. California, Riverside	Fresh
19		Kinnow[f]	California	Univ. California, Riverside	Fresh
20		Honey[f]	California	Univ. California, Riverside	Fresh
21	Lemon-orange hybrid	Not assigned[g]	California	Univ. California, Riverside	Fresh

[d] Cross between Dancy mandarin x Mediterranean sweet orange.
[e] Cross between King orange x Satsuma mandarin.
[f] Cross between King orange x Willow Leaf mandarin.
[g] Cross between Tetraploid Lisbon lemon x sweet orange (Trovita).

Identification of constituents. The identification of the nitrogenous constituents in citrus juices was based upon: a, congruence of ninhydrin color reactions and R_F values obtained from chromatograms of pure compounds, citrus juices, and mixtures of pure compounds and citrus juices; and b, specific or distinctive confirmatory tests as described below. At least 2 different pairs of solvents were employed for two-dimensional chromatograms of all citrus juices and at least 3 replicate chromatograms were developed with each solvent pair.

Confirmatory tests. The tests employed for confirmation of the nitrogenous constituents in orange juice are shown in Table 2. Because of the similarity in chromatographic patterns of orange juice and other citrus juices and the uniformity of color reactions and R_F values obtained with the small-scale, two-dimensional chromatography procedure, all of the confirmatory tests were not applied to all of the citrus juices. Constituents for which satisfactory confirmatory tests on filter paper chromatograms have not been previously reported were confirmed by the following procedures:

Asparagine and glutamine. Pure asparagine, as well as citrus juices, produced a yellow to tan spot, which turned purple after standing or after treatment with HCl fumes, at identical positions on 2-dimensional chromatograms developed with 3 different pairs of solvents and stained with ninhydrin reagent. Since asparagine and various peptides *(18)* yield similar color reactions with ninhydrin and HCl fumes, the following procedure was employed to further establish the presence of asparagine as well as glutamine in orange juice: To 1-ml. aliquots of single-strength orange juice and 0.06 molal solutions of asparagine and glutamine, respectively, was added 0.1 ml. of concentrated hydrochloric acid. The solutions were heated for 4 hours at 90°C. and aliquots of each of the untreated and hydrolyzed materials were individually chromatographed as previously described with solvents D and E. The control and hydrolyzed solutions of asparagine each gave single spots with R_F values corresponding with asparagine and aspartic acid, respectively. The control and hydrolyzed solutions of glutamine also each produced only one spot, corresponding to glutamine and glutamic acid, respectively. The untreated citrus juice yielded characteristic purple and tan spots with R_F values corresponding to glutamine and asparagine, while the purple spot and the tan spot occupying the areas corresponding in R_F values with glutamine and asparagine, respectively, did not appear, and the aspartic acid and glutamic acid spots were intensified on chromatograms of the hydrolyzed orange juice.

Glutamic acid and glutamine. Olcott *(25)* has shown that within the pH range 2.5 to 4, 92 to 96% of glutamic acid is converted to pyrrolidonecarboxylic acid after 4 hours of autoclaving at 125°C. Pure glutamic acid solutions as well as most citrus juices (Table 5) are within this pH range and may be autoclaved without further treatment to destroy a large percentage of the free glutamic acid. Pure glutamine autoclaved for 4 hours in neutral or slightly acidic (pH 3.5) solution is also converted to pyrrolidonecarboxylic acid.

An aliquot of orange juice was autoclaved for 4 hours at 125°C. and chromatographed with solvents D and E, respectively, as previously described. Chromatograms of an untreated sample of orange juice contained characteristic purple spots with R_F values corresponding to pure glutamine and glutamic acid. Chromatograms of the autoclaved orange-juice sample were devoid of color at the positions normally occupied by glutamine, and only a slight trace of color remained at the position corresponding to glutamic acid.

Serine. Consden *(5)* has suggested that, when Nessler's reagent is added to solid periodic acid until the precipitate first formed is just dissolved, the resulting solution may be used to identify serine and threonine. In the authors' hands this reagent did not prove to be satisfactory for the identification of serine on small-scale, two-dimensional chromatograms. However, since serine and other hydroxy-amino acids are preferentially oxidized by periodate *(27, 40)* it was possible to confirm the presence of serine in orange juice by comparison of two-dimensional chromatograms of periodate-treated and untreated orange juice.

Five-ml. aliquots of single-strength orange juice and a 0.06 molal solution of serine, respectively, were adjusted to pH 10 with concentrated KOH, 70 mg. of solid KIO_4 were added, and the solutions were heated at 90°C. Aliquots of each solution were removed at 10, 30, 50, and 70 minutes and chromatographed with solvents D and E as previously described. The chromatograms of the treated solutions showed a progressive

TABLE 2

Color tests employed for the identification of nitrogenous constituents in citrus juices on small-scale two-dimensional filter paper chromatograms

Compound	Ninhydrin reaction		Confirmatory test			
	Color	Approximate sensitivity	Reagent or procedure	Result	Approximate sensitivity	Reference
		gamma			*gamma*	
Alanine	Purple	0.5	.....	.....	.....	.....
α Aminobutyric acid	Purple	1	Rate of ninhydrin color formation	See text	1	Present authors
Arginine	Purple	1	Sakaguchi, modified	Red color	2(0.5[b])	31
Asparagine	Yellow-tan	1	Acid hydrolysis	Formation of aspartic acid	2	Present authors
Aspartic acid	Blue-purple	1	Bromeresol green	Yellow spot on blue background	3	31
Glutamic acid	Purple	0.5	Conversion to pyrrolidonecarboxylic acid	Disappearance of ninhydrin spot	0.5	Present authors
Glutamine	Purple	0.5	Conversion to pyrrolidonecarboxylic acid Acid hydrolysis	Disappearance of ninhydrin spot Formation of glutamic acid	0.5 0.5	Present authors Present authors
Histidine	Grey-green	3	Diazotized sulfanilic acid	Red-orange color	0.5[b, c]	2
Proline	Yellow	1	Isatin	Blue color on yellow background	1	31
Serine	Purple	0.5	Periodate oxidation	Disappearance of ninhydrin spot	1	Present authors

[b] One-dimensional test tube chromatogram (*30*) developed with solvent D.
[c] A satisfactory color test could not be obtained if phenol was employed as a solvent.

decrease in purple color at the position corresponding to serine. Only a trace of serine could be observed in the oxidized orange-juice sample after 70 minutes at 90°C. Alanine, aspartic acid, glutamic acid, proline, and γ-amino butyric acid were not significantly affected by the periodate reaction. However, asparagine, glutamine, and arginine could not be detected in periodate-treated orange juice after 30 minutes at 90°C. The loss of asparagine and glutamine was expected because of the alkalinity of the reaction mixture. The very rapid destruction of arginine was not anticipated, since it has been reported that only tryptophan, methionine, cystine *(22)*, and histidine *(40)* will react with periodate under the conditions employed for the oxidation of hydroxy-amino acids.

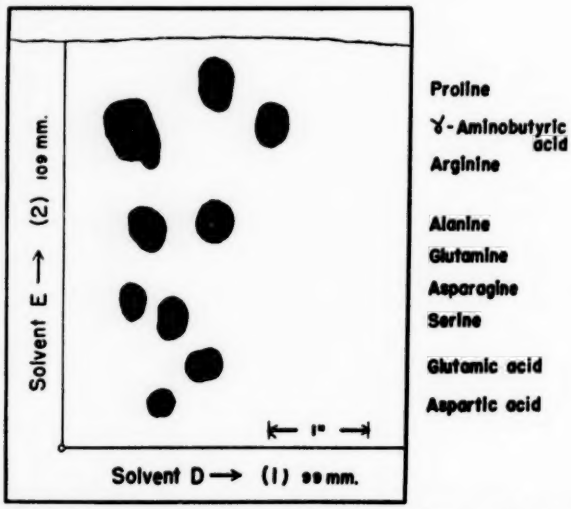

Figure 1. Tracing of a small scale two-dimensional filter paper chromatogram of California Valencia orange juice. Filter paper: Schleicher & Schuell No. 589 Blue Ribbon. Solvent D: tert.-butanol-water-formic acid. Solvent E: phenol-water-ammonia.

γ-Aminobutyric acid. Two-dimensional chromatograms of most of the citrus juices contained a purple spot which developed slowly (about 60 minutes) at room temperature with ninhydrin reagent and which did not coincide with the R_F values of any of the known, naturally occurring amino acids. Sarcosine developed at about the same rate as the unknown compound, but its R_F value in solvent D was lower than the unknown. Mixed chromatograms of sarcosine and orange juice confirmed its non-identity. The position of the unknown on two-dimensional chromatograms developed with solvents D and E, respectively, suggested that it might be one of the aminobutyric acids. Both α-aminoisobutyric acid and β-aminoisobutyric acid had the same R_F values as the unknown, but did not give a color reaction with ninhydrin unless heated at 95°C. for several minutes. α-Amino n-butyric acid had a lower R_F value in solvent D and gave an intense purple color with ninhydrin in 10 minutes at room temperature. γ-Aminobutyric acid had identical R_F values and ninhydrin staining reactions as the unknown. Although β-amino n-butyric acid was not available for testing, and therefore cannot be eliminated as a possibility, the occurrence of this compound in nature has not been reported. On the other hand, γ-aminobutyric acid has been reported in extracts from both plant and animal sources *(1, 15, 17, 36, 38, 39, 41)*. Since appreciable quantities of glutamic acid and glutamine, probable precursors of γ-aminobutyric acid *(1, 38)*, are present in citrus juices (Table 5) it is suggested that γ-aminobutyric acid is a normal constituent in citrus juices.

TABLE 3

Reproducibility of R_{ISF} values obtained from two-dimensional chromatograms of California Valencia orange juice

Compound	Solvent D							Solvent E						
	Replicates, single sample				20 orange juice samples[1]			Replicates, single sample				20 orange juice samples[1]		
	1	2	3	Av.	Av.	Range	Standard deviation	1	2	3	Av.	Av.	Range	Standard deviation
Alanine	43	46	44	44	43	39-47	2.6	57	59	60	59	58	56-60	1.3
α-Aminobutyric acid	56	55	62	58	57	53-62	2.7	78	78	78	78	80	78-82	1.5
Arginine	22	26	23	24	23	19-30	3.2	83	81	82	82	81	75-84	2.8
Asparagine	20	19	19	19	20	17-22	1.9	38	40	37	38	38	35-41	2.3
Aspartic acid	23	24	25	24	26	22-30	2.4	13	16	14	14	14	12-16	1.0
Glutamic acid	41	42	39	41	38	33-42	3.1	24	24	23	23	23	22-25	1.3
Glutamine	23	21	25	23	23	21-25	1.2	51	54	56	54	55	51-58	2.1
Proline	42	46	47	45	44	40-48	2.5	91	92	90	91	91	88-93	1.2
Serine	32	30	30	31	30	26-33	2.1	36	34	33	34	33	30-36	1.9
						σ, average	2.4						σ, average	1.7

[1] Each of the 20 individual values used to obtain the average was an average of three replicate values obtained for each sample.

RESULTS AND DISCUSSION

A tracing of a typical small-scale, two-dimensional, filter-paper chromatogram of orange juice is shown in Figure 1. The reproducibility of R_F values obtained from 2-dimensional chromatograms of the nitrogenous constituents in orange juice are shown in Table 3. In Table 4 are shown the R_F values found for the nitrogenous constituents in fresh California Valencia orange juice, in mixtures of orange juice and pure compounds, and for pure compounds chromatographed singly. It may be seen that the tert.-butanol : water : formic acid mixture, employed as the first solvent (D), yields somewhat less precise R_F values than the phenol : water : NH_4OH solvent (E) used for development in the second direction. However, the high precision of both sets of R_F values found for pure compounds and the same compounds present in citrus juices would appear to oppose the view *(9)* that R_F values are not sufficiently reproducible to be considered as a reliable criterion of identity.

TABLE 4

R_{two-F} values obtained from two-dimensional chromatograms of orange juice, pure compounds, and mixtures of orange juice and pure compounds developed with two different solvent systems [a]

Constituent	Solvent A [1], Solvent B [2]			Solvent D [1], Solvent E [2]		
	Orange juice	Orange juice plus pure compound	Pure compound	Orange juice	Orange juice plus pure compound	Pure compound
Alanine	34, 54	31, 52	36, 53	44, 58	44, 59	47, 59
γ-Aminobutyric acid	....	....	....	58, 78	57, 79	55, 78
Arginine	6, 19	6, 18	3, 19	24, 82	22, 84	21, 84
Asparagine	17, 39	....	14, 40	18, 41	18, 41	20, 39
Aspartic acid	....	....	....	24, 14	25, 15	32, 15
Glutamic acid	27, 74	26, 76	33, 72	35, 24	37, 24	42, 25
Glutamine	....	....	....	23, 55	24, 56	22, 53
Proline	40, 86	42, 88	43, 88	41, 91	46, 90	48, 93
Serine	22, 33	26, 32	26, 32	30, 34	31, 34	31, 34

[a] Each value is the average of 3 replicate chromatograms. The solvent designations are given in the experimental section. The numbers enclosed by brackets refer to the order in which the solvents were used.

The nitrogenous constituents found in various citrus fruit juices are shown in Table 5. The relative amounts of each constituent were roughly estimated by visual comparison of their spot intensities on two-dimensional chromatograms developed with solvents D and E, respectively. The arginine content of Sample 2 (orange juice) was estimated as 75 ± 25 mg./100 ml. This value was obtained by visual comparison of spot areas and intensities *(3)* on two-dimensional standard and sample chromatograms developed with solvents D and E, respectively, and stained with ninhydrin, as well as one-dimensional chromatograms of the same materials developed with solvent D and stained with a modified Sakaguchi reagent *(31)*. The histidine content of the same orange juice sample was similarly estimated as a maximum of 5 ± 3 mg./100 ml. based on one-dimensional chromatograms of standard and sample developed with solvent D and stained with diazotized sulfanilic acid reagent *(2)*.

TABLE 5

Some nitrogenous constituents in fresh and processed citrus fruit juices [1]

Type	Orange					Tangerine		Grapefruit					Lemon			Lime	Tangor	Mandarin			Lemon-orange
Variety	Parson Brown	Valencia		Navel		California	Commercial	Marsh, Ariz.	Marsh, Calif.	Pink, Texas		Marsh, Fla.	Eureka	Commercial		Lime	Dweet	Kara	Kinnow	Honey	Sweet orange × tetraploid Lisbon lemon
Sample No.[m]	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Condition[n]	F	F	C	F	C	F	C	F	F	F	C	C	F	C	C	F	F	F	F	F	F
Constituent																					
Aspartic acid	3	3	4	4	3	4	3	4	3	4	4	4	3	4	3	2	3	3	2	2	3
Asparagine	2	2	3	3	3	3	1	3	2	3	2	3	1	3	1	1	3	2	2	1	1
Serine	2	3	4	3	3	3	2	3	3	2	3	3	3	3	3	3	2	3	3	3	3
Alanine	2	2	2	2	2	2	1	3	2	3	3	3	2	2	2	3	2	2	2	2	2
Glutamic acid	1	2	1	2	1	2	1	2	2	2	2	2	2	2	2	3	3	2	2	2	3
Proline	2	4	2	1	2	1	0	2	1	1	1	1	1	1	1	2	4	3	3	3	3
γ-Aminobutyric acid	2	3	2	1	1	2	0	2	2	2	3	2	1	1	1	1	3	2	0	0	2
Glutamine	1	1	0	1	0	1	0	1	1	1	tr	0	0	0	0	0	1	1	1	0	0
Arginine	2	4	4	2	3	3	1	3	2	3	3	3	0	0	0	0	3	3	3	3	2
Histidine	....	tr	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....
pH	4.2	3.7	3.5	3.4	3.5	3.6	3.4	3.0	2.8	3.4	3.2	3.0	2.1	2.2	....	2.2	3.3	3.1	3.1	3.6	2.4
Acid	0.39	1.11	1.20	1.12	0.99	0.74	0.76	1.70	2.15	0.93	1.31	1.63	6.58	5.47	....	7.88	1.12	1.56	1.51	0.99	5.86
Brix	12.8	12.2	13.0	12.9	14.7	13.0	13.7	10.6	13.0	9.9	12.7	12.2	9.7	8.5	....	12.2	12.2	18.3	16.6	15.9	11.9
Brix-acid ratio	33	11	11	13	15	18	18	6.2	6.1	11.0	9.7	7.5	1.5	1.6	....	1.6	11.0	12.0	11.0	16.0	2.0

[1] The numbers indicate the relative amounts of constituents estimated by visual comparison of spot intensities on two-dimensional filter paper chromatograms. The number 0 indicates that a constituent could not be detected and that less than approximately 5 mg. percent of the constituent was present.

[m] The sample numbers are the same as shown in Table 1.

[n] F and C refer to fresh and canned, pasteurized juices, respectively.

It may be seen from Table 5 that with the exception of grapefruit and tangerine, the juice of each type of mature citrus fruit appears to have a characteristic qualitative and quantitative "pattern" of nitrogenous constituents. Preliminary studies on the nitrogenous compounds in the juice of oranges at different stages of maturity indicate that the amounts of several amino acids vary considerably with the maturity of the fruit. A study of the relationship between the maturity of California Valencia oranges and the nitrogenous constituents in the juice will be reported at a later time. It is of interest that the number and relative amounts of the nitrogenous constituents did not appear to vary appreciably with the geographical origin of the fruit. A complete summary of the nitrogenous constituents reported in citrus fruit juices is shown in Table 6. The present study corroborates earlier, scattered reports of the presence of relatively large amounts of a wide array of amino acids and related compounds in citrus fruit juices. Putrescine has been reported to be present in orange *(13)* and grapefruit *(14)* juices. However, this compound was not detected on ninhydrin stained chromatograms of either juice, indicating that less than 0.01% of putrescine was present in the citrus juices examined by the present authors. It was not possible to test the citrus juices for stachydrin, choline, and betaine because of the unavailability of satisfactory procedures for determining the location of these compounds on filter paper chromatograms. Although glutamine was found in several other citrus juices, this compound could not be detected on chromatograms of lemon juice. Since Scurti and Plato *(32)* reported the isolation of glutamine from lemon juice, it is estimated that less than 0.005% glutamine may occur in the juice of California lemons. With the possible exception of glutamine, only small differences in the nitrogenous constituents were observed between fresh and commercially processed citrus juices. A quantitative study is in progress on the changes in the nitrogenous constituents of orange juice during processing and storage. No protein or soluble peptides were detected on filter paper chromatograms of citrus juices, corroborating Smith's *(33)* conclusion that orange juice does not contain soluble, proteinaceous material.

SUMMARY

Small-scale filter-paper chromatography procedures have been applied to the determination of the nitrogenous constituents in the filtered juices of orange, tangerine, grapefruit, lemon, lime, kara, kinnow, honey, dweet, and a lemon-orange hybrid. In addition to asparagine, aspartic acid, glutamine, arginine, and histidine, which have been reported previously, alanine, serine, glutamic acid, and proline have been detected in orange juice for the first time. Many of these same compounds have been found in all of the citrus fruit juices studied. Evidence has been presented to support the conclusion that γ-aminobutyric acid is a constituent in all commercial citrus fruit varieties.

The character and quantities of the free amino acids and related compounds present in the juice of most of the citrus fruits appear to be characteristic of the variety and independent of the geographical origin of the fruit.

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The authors are indebted to Dr. M. S. Dunn and Dr. R. B. Henderson for generous gifts of authentic samples of γ aminobutyric acid and β aminoisobutyric acid, respec-

TABLE 6

Summary of nitrogenous constituents in citrus fruit juices [a]

Constituent	Orange		Tangerine	Grapefruit	Lemon	Lime	Dweet Tangor	Mandarin			Lemon-orange hybrid
	Valencia	Navel						Kara	Kinnow	Honey	
Alanine	A	A	A	A	A	A	A	A	A	A	A
γ-Aminobutyric acid	A	A	A	A	A	A	A	A	O	O	A
Arginine	21, A	A	A	A	O	O	A	A	A	A	A
Asparagine	21, 32, A	A	A	A	34, A	A	A	A	A	A	A
Aspartic acid	21, A	A	A	A	A	A	A	A	A	A	A
Betaine	43	....	....	....	....	....	....	....	....	....	....
Choline	21, 37	....	....	....	....	....	....	....	....	....	....
Cysteine	16, 20	16, 20	....	20	20	20	....	....	....	....	....
Glutamic acid	A	A	A	A	A	A	A	A	A	A	A
Glutamine	32, A	A	A	A	32, O	O	A	A	A	O	O
Glutathione	6, 16, 20	16, 20	....	20	20	20	....	....	....	....	....
Histidine	21, A	....	....	....	....	....	....	....	....	....	....
Proline	A	A	A	A	A	A	A	A	A	A	A
Putrescine	13	....	....	14	....	....	....	....	....	....	....
Serine	A	A	A	A	A	A	A	A	A	A	A
Stachydrin	21, 43	....	....	14	....	....	....	....	....	....	....

[a] The numbers refer to literature citations. A indicates the presence of constituent based on the present authors' investigations. O indicates less than 5 mg./100 ml. of juice.

tively, to Dr. F. M. Turrell for a translation of Hiwatari's *(14)* paper, and to the University of California at Los Angeles and Riverside for samples of various citrus fruits of known history.

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THE DIETHYLSTILBESTROL CONTENT OF THE MEAT FROM CHICKENS TREATED WITH THIS ESTROGENIC SUBSTANCE[a]

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Use of the synthetic estrogenic compound diethylstilbestrol (4,4′-dihydroxy-α,β-diethylstilbene) has gained considerable popularity among poultry producers for fattening cockerels. Because this compound has female sex hormone activity in man it seemed desirable to determine, if possible, the amount of carryover of diethylstilbestrol in the meat of cockerels treated with this substance.

Bird, Pugsley, and Klotz *(1)* used bioassay techniques to show that the muscle of treated birds possessed low estrogenic potency whereas lipid tissue possessed much greater activity. Gowe *(7)* observed that 1 ml. of fat from a treated tom turkey was sufficient to elicit estrogenic activity in rats. Because of recent advances in chemical methods of estimating relatively small amounts of diethylstilbestrol, it has been possible to determine this substance in the necks, liver, and breast muscles plus abdominal fat of treated and untreated chickens.

For this study a group of 12 ten-week-old cockerels was divided into two groups of 6. One group was used as a control and the other was treated by placing subcutaneously in the neck at the base of the head a fat soluble pellet containing 15 mg. of diethylstilbestrol.[b] After 4 weeks the birds were killed, dressed, and frozen. An additional 4 cockerels were obtained for study from a commercial source. These birds had been treated with a subcutaneous injection in the neck of an aqueous pasty suspension also containing 15 mg. of the estrogen.[c] After 31 days these birds were killed, dressed, and frozen. Usually poultrymen market birds 4 to 6 weeks following hormone treatment. The analyses were all completed within 2 weeks post mortem. All chickens had been on excellent quality commercial rations and weighed on the average 3.5 pounds.

METHODS

Exploratory work indicated that tissue extraction with acetone followed by colorimetric determination by use of the Folin-Denis phenol reagent might be used for diethylstilbestrol determinations. However, the large amount of interfering substances extracted from tissue by acetone gave trouble. The color reaction of Dingemanse *(3)* and Dingemanse and Tyslowitz *(4)* using antimony pentachloride seemed promising in view of the report of Teaque and Brown *(10)*, and of Warren *et al. (11)*. From these

[a] Published with the permission of the Associate Director of the Ohio Agricultural Experiment Station, Journal Article No. 26-52.

[b] Pellets were obtained from the American Scientific Laboratory, Madison, Wisconsin, and were implanted as directed by the manufacturer.

[c] Preparation produced by Maddox and Moore, Inc., Indianapolis, Indiana, and used according to their directions.

reports and that of Dodgson *et al. (5)* it appeared advisable to attempt to determine the glucuronide of diethylstilbestrol as well as the free compound. The method which was found satisfactory follows.

The necks, livers, and breast muscle together with abdominal fat from each bird were treated separately. After thawing, each specimen was cut into small pieces and mixed with 150 ml. acetone using a Waring blender. The acetone was decanted. This extraction was repeated 3 times. The total acetone extract was filtered and the residue was washed with two 50-ml. portions of acetone. The filtered extracts and washings were combined and the acetone removed by distillation under reduced pressure (water pump). The residue was taken up and transferred to a separatory funnel, using 100 ml. of a mixture of 35 parts peroxide free ethyl ether and 65 parts petroleum ether. The glucuronide fraction was removed by washing with four 75-ml. portions of 9% sodium bicarbonate. These extracts were pooled and back washed with two 50-ml. portions of the ether mixture. These were added to the original ether solutions. The bicarbonate solution was brought to a pH of approximately 2, using pH paper, by the addition of 12 N. phosphoric acid. Hydrolysis of the glucuronide was carried out by refluxing the acid solution 2½ hours. After cooling, this solution was extracted with four 75-ml. portions of peroxide free ethyl ether. The ether was then evaporated and the residue was taken up in 30% alcohol and filtered into a 25- or 50-ml. volumetric flask and made to the mark.

The conditions of hydrolysis given here differ somewhat from that of Teaque and Brown *(10)* who recommend 90 minutes at pH 3.45 and 180°C. Since Dodgson *et al. (5)* indicate that the glucuronide is easily hydrolyzed by acid it is reasonable to say that if there was much combined diethylstilbestrol in the tissue it would have been detected. It is unfortunate that it was impossible to carry out the precise directions of Teaque and Brown or suitable pilot experiments.

The original ether extract containing the free diethylstilbestrol was washed 5 times with 50-ml. portions of 10% sodium hydroxide solution. The combined alkaline extracts were immediately acidified to Congo Red using 12 N phosphoric acid and then extracted, using four 75-ml. portions of peroxide free ethyl ether. The ether was evaporated and the residue was taken up in 30% alcohol. The alcohol was filtered into volumetric flasks and filled up to the mark. The liver, neck, and breast-abdominal fat residues were made to 50-ml., 250-ml., and 25-ml. volumes, respectively.

For all determinations 1.0 ml. of the 30% alcohol solutions was used for color development both for standards and unknown. Standards were prepared containing 1, 2.5, 5, 10, 15, 20, 25, 30, 40, and 45 micrograms of diethylstilbestrol per ml. of 30% alcohol.

One ml. of the alcohol solution was placed in a 200-ml. Erlenmeyer flask. The alcohol was allowed to evaporate in an electric oven at 100°C. This required 20 minutes and longer times were avoided since some loss of the diethylstilbestrol might result. For this reason larger aliquots than 1 ml. of the alcohol solution were not desirable. After drying, the flask was cooled in water. To the flask was added 10 ml. of antimony pentachloride solution (3 ml. of antimony pentachloride diluted to 100 ml. with ethylene dichloride). After allowing 15 minutes for maximum color development, the color was measured in an Evelyn photoelectric colorimeter using the 540-mμ filter.

RESULTS AND DISCUSSION

Table 1 shows the data for the standard curve upon which these determinations were based. It is quite evident that Beer's law holds up to 30 γ of diethylstilbestrol.

Before working on the experimental birds a series of experiments were conducted to determine the amount of diethylstilbestrol recoverable from known additions to liver tissue. Two mg. was added to each of 5 livers. By following the above procedure the percentages recovered were 81.0, 81.5, 82.5, 83.7, and 86.0 for an average recovery of 82.9%. This had been taken into account by assuming 80% recovery and computing the final results on that basis.

TABLE 1

Color development in relation to standards

Micrograms of diethylstilbestrol per ml. of standard	Optical density
1	0.0327
2.5	0.0706
5	0.138
10	0.286
15	0.417
20	0.553
25	0.663
30	0.824
40	1.059
45	1.155

The diethylstilbestrol content, either free or combined, in the livers and breast tissue plus abdominal fat in both the control and experimental birds were all below the amount detectable by this method of analysis. This indicates that for the livers, average weight 32 g., there was less than 62.5 γ of diethylstilbestrol present in either form. Consequently, the livers contained less than 125 γ total and perhaps much less.

Similarly in the breast muscle plus abdominal fat, average weight 165 g., all birds contained less than 31.25 γ in the free or combined form. This means that the breast plus abdominal fat in no bird, control or treated, exceeded 62.5 γ total diethylstilbestrol.

Somewhat different results were obtained from analyses of the necks, average weight 53 g., where the diethylstilbestrol was injected. Of course, no estrogen was found in the control. (The limits of the method were as for the breast plus abdominal fat 31.25 γ for free or combined, or 62.5 γ total). However, there were rather large residues in the necks from birds treated with the fat soluble pellet. Table 2 shows the results of the remaining estrogen in the necks of the birds.

TABLE 2

Diethylstilbestrol in the free form in the necks of cockerels receiving subcutaneous pellet implants

Bird number	Micrograms diethylstilbestrol
1	4,630
2	3,000
3	4,060
4	2,630
5	4,380
6	5,130
Mean	3,970

In the necks of the 4 birds commercially treated with a viscous aqueous suspension of diethylstilbestrol the residues of free estrogen found were: 700 γ in one, 335 γ in another, and less than 31.25 γ in two. As in the other necks less than 31.25 γ of the compound occurred in the combined form.

If one surveys the clinical use of diethylstilbestrol, it is apparent the magnitude of the pellet residues is such that some effect on a consumer might result if these residues were eaten in toto (cf. 2, 6, 8, 9). Consequently, it is important that the pellet be placed in such a way that upon dressing the bird any residual diethylstilbestrol will not remain on the carcass sold to the consumer. When the implants are placed at the base of the head no problem should result since this portion of the carcass should be discarded. Furthermore, implants should not be made so that residues will be found in the meaty portions of the carcass which are consumed.

It appears that the aqueous preparation used in this study left a much smaller amount of estrogen in the neck and consequently the danger of undesirable carry-over is somewhat lessened. However, from the practical poultryman's point of view, there is the unanswered question as to whether the diethylstilbestrol has been dissipated too early to produce the maximum fattening of the birds. Further work along this line would be desirable.

SUMMARY

In order to obtain some information on the amount of hormone remaining in chickens treated with diethylstilbestrol a method has been developed for the determination of diethylstilbestrol in the necks, livers, and muscle tissue of chickens.

No significant amounts of diethylstilbestrol were detected in the livers and breast muscle plus abdominal fat of 6 chickens receiving 15 mg. of diethylstilbestrol by fat type pellet and 4 chickens receiving 15 mg. by aqueous pasty suspension.

The pellet residues in the necks (site of implantation at the base of the heads) of the 6 chickens averaged 3,970 mg. of diethylstilbestrol whereas the residue from an aqueous type preparation averaged in four chickens approximately 280 mg. with 2 out of the 4 having no detectable amounts present. These results indicate that hormone implants should be placed in the bird in such a way that upon dressing the site of implantation will be discarded and not reach the consumer.

Acknowledgment

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SPORE GERMINATION, GROWTH, AND SPORE FORMATION BY *CLOSTRIDIUM BOTULINUM* IN RELATION TO THE WATER CONTENT OF THE SUBSTRATE[a]

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Some work on the growth of bacteria at different moisture levels in different materials has been done, as, for one example, that by Haines and Elliot *(7)*, and mention, or discussion, of the significance of water as a factor in the growth of microorganisms occurs in numerous places [see Barry *(2)*, Fabian *(4)*, and especially Haines *(6)*]. Little seems to be known, though, about the water requirements for the germination, or formation, of bacterial spores, although the subject has both theoretical and practical interest. Considerable work has been done on the relationship between moisture and germination of fungus spores [see Gottlieb *(5)* for references], but it is not likely that these data apply to bacterial spores since it is generally accepted that fungi are less exacting in their moisture requirements than bacteria.

In most investigations of the effect of moisture on the growth of microorganisms it has been recognized that the determining factor was the free moisture, or moisture activity, as established by determining the water vapor pressure in equilibrium with the substrate. Different substrates show different equilibrium vapor pressures at the several moisture levels so that it is necessary to establish values for each. This is usually done by exposing portions of the substrate which have been adjusted to different moisture concentrations to fixed relative humidities in closed systems, and weighing at intervals to determine whether there is a change in weight at equilibrium. The humidity of the closed system is controlled either by sulfuric acid-water mixtures, or by saturated solutions of different salts. Each method of control is based upon the fact that there is a definite and different relative humidity above the solutions of different concentrations of chemical substances at any given temperature. By the proper selection of a series of concentrations of sulfuric acid or of saturated solutions of common salts it is possible to attain readily and easily a wide range of humidities for a certain temperature. The latter method has the advantage that an excess of a salt in a saturated solution in a closed environment will give a constant relative humidity which is self-adjusting indefinitely following opening and closing of the container. Once the enclosed system has reached equilibrium the solution in contact with the solid crystals remains saturated, and the humidity of the gas in contact

[a] This paper reports research undertaken in cooperation with the Quartermaster Food and Container Institute for the Armed Forces, and has been assigned Number 396 in the series of papers approved for publication. The views or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the Department of the Army.

with the liquid remains constant, no matter what the relative volumes of the solid, liquid, and gaseous phases may be.

The observations which have been made by different workers on bacterial growth at different substrate moisture concentrations are not strictly comparable because of different procedures of experimentation, differences in the substrates utilized, and dependence on the natural, and consequently heterogenous, flora. However, there seems to be general agreement that growth is slight, or unlikely, at a relative humidity below 90% [Schmidt *(8)*, Haines and Elliot *(7)*, and Barry *(2)*], although Haines *(6)* noted growth on lean meat held in still air at a relative humidity as low as 70%. Ulrich and Halvorson *(9)* investigated the effect of moisture on the possibilities of growth of *Clostridium botulinum* in canned bread. Assuming that growth was restrained at a moisture concentration of 32% they established that at 37°C.(98.6°F.) the corresponding equilibrium vapor pressure is in the range of 92 to 94%.

Dack and Segalove (Dack, *3*) inoculated spores of *Cl. botulinum*, type A, into samples of dehydrated beef and pork adjusted to moistures of 10, 20, 25, 30, and 40%, and tested for toxin as an indicator of germination and growth at intervals up to 9 months. Toxin could be demonstrated only in the 40% samples. The equilibrium vapor pressure of the various moisture concentrations was not reported.

The investigation here reported was undertaken to establish the relationship between moisture and spore germination, spore formation, and growth of *Clostridium botulinum*, as a part of a broad program of investigation of the biology of bacterial spores.

EXPERIMENTAL METHODS

Organism. *Clostridium botulinum*, strain 62A, was used in all experiments. The authenticity of the culture was established by toxicity tests on guinea pigs with a 10-day culture. The organism was grown in difco brain-heart infusion broth with a thioglycollate supplement. Vegetative cell suspensions were prepared from 24-hour cultures by centrifuging out the cells, washing 4 times, resuspending and standardizing by Petroff-Hauser counts. Spore suspensions were prepared similarly from 10- to 15-day cultures, and were heated at 75°-80°C.(167°-176°F.) for 30 minutes to destroy the vegetative cells.

Substrate. The substrate was prepared from trimmed beef liver by grinding finely, drying in a thin layer at 55°C.(131°F.), and powdering in a ball mill. The residual moisture was between 12 and 20% with different lots. The liver powder was sterilized with ethylene oxide under a partial vacuum. In some of the experiments the liver was infused before drying, and the infusion used in rehydration. No difference in results between raw and infused liver was noted.

Procedure. The moisture content of the liver powder was determined by drying to constant weight in the usual manner. A portion was weighed into a sterile beaker and the amount of liquid to be added, in order to have the desired final moisture level, was calculated. This was added as spore suspension, sodium hydroxide solution to adjust to pH 7.5 as determined by preliminary titration, and M/15 phosphate buffer to complete the required quantity of liquid. Mixing was effected with a sterile spatula, and the inoculated substrate was filled into a device for filling semi-solid materials into thermal death time tubes developed by O. B. Williams and J. Yesair at the Research Laboratory of the National Canners Association in 1940. This device, illustrated in Figure 1,[b] is a cylinder fitted with a screw actuated plunger, which discharges through a long slender nozzle. The entire device can be sterilized by steam. About 2 g. of the rehydrated

[b] The illustration was done by W. W. Milburn, Jr.

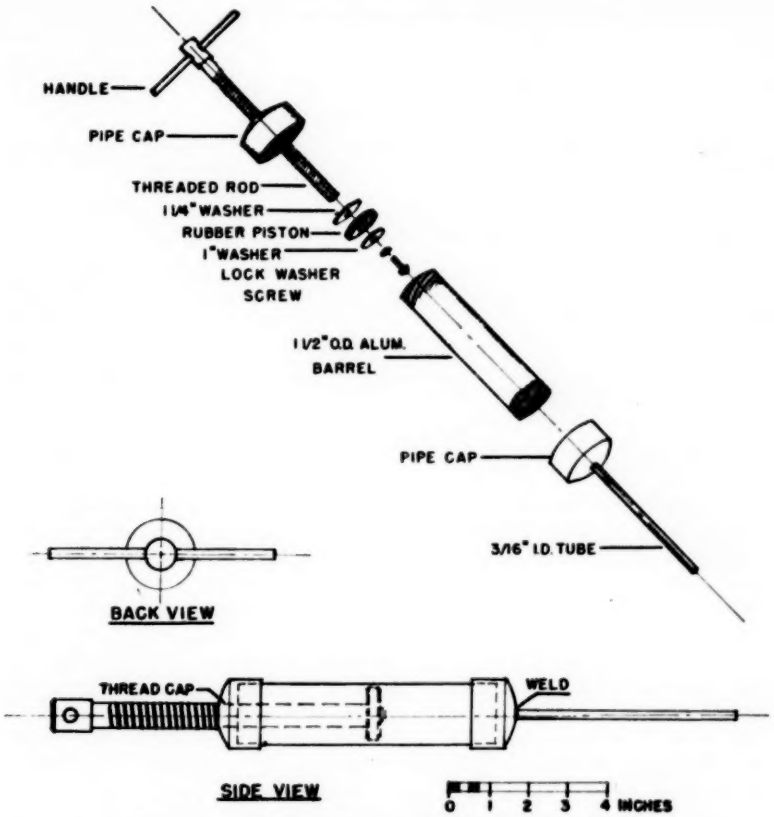

Figure 1. Device for filling semisolids into small tubes.

inoculated liver was filled into 10-cm. pyrex tubes of 9 mm. inside diameter. A strip of detinned base plate was forced down into each tube to promote anaerobiosis, the tubes were placed in a vacuum desiccator and exhausted under negative pressure for between 30 minutes and an hour. The vacuum was released with natural gas, the tubes sealed immediately in a blast lamp and placed under incubation at 37°C.(98.6°F.). At intervals tubes were opened and emptied into sterile tared weighing bottles. The medium was weighed and sufficient water added to make a dilution of 1-10.

Serial dilutions were made, and counts of total cells and spores were made in oval tubes in the pork medium of Yesair *(10)*, or the medium of Ayres *(1)*. The initial count was usually in the range of from 1,000 to 2,000 per gram of substrate.

Portions of each lot were taken for total moisture determination and for moisture activity, which was determined by placing aliquots in desiccators over saturated solutions of salts selected to give different water vapor pressures, and determining equilibrium by weighing at intervals for change of weight.[c] An approximate rather than an exact equilibrium value was ordinarily recorded.

[c] We are indebted to Dr. Charles I. Phillips for calling to our attention this method of control of relative humidity. Further details may be found in International Critical Tables, 1, 67-68, and in the article by Sweetman, Ecology, **14**, 40-45, 1933.

Considerable irregularity in results was experienced in the early stages of the work in that an experiment might yield negative results, or a successful experiment could not be repeated. Eventually it was found that changes in pH of the liver to a value such that *Cl. botulinum* would not grow was the cause of the trouble. This difficulty was overcome by making the liver tissue slightly alkaline at the time of rehydration, and by including phosphate buffer as one of the rehydrating liquids.

Attempts to duplicate the results with whole egg powder as the substrate were not successful because a constant moisture level could not be maintained throughout the powder. Most of the moisture was pressed out with the first few tubes filled.

EXPERIMENTAL RESULTS

Rapid growth, and spore formation as customarily demonstrated, from both vegetative cell and spore inocula took place in liver with 60% moisture and corresponding relative humidity at 37°C.(98.6°F.) of between 97 and 100%. At 50% moisture with relative humidity between 96 and 98%, maximum growth likewise was attained rapidly, but spore formation was somewhat diminished in amount, and with the maximum count being reached only after a longer incubation period than usual, up to as much as 34 days, as compared with 4 days with some of the 60% moisture experiments.

The effect on growth and spore formation of moisture concentrations of 45% with a relative humidity of 94-96% was very striking. Rate and amount of growth and spore formation were sharply reduced. Maximum total counts were recorded only after as much as 3 weeks of incubation, at most were not more than about one-third as great as those obtained at 60% moisture, and frequently were much less. Spore formation was irregular, with maximum values being recorded after from 7 to 22 days, and with counts ranging from 0 to as much as 340,000 per gram of substrate. The 45% moisture data suggest that growth without spore formation can take place in a medium which is satisfactory except for moisture deficiency, and thus tend to emphasize further that growth and spore formation by sporulating bacteria do not necessarily parallel each other.

As the limiting moisture concentration for growth was approached, irregularity in results became more pronounced. At 40% total moisture, with relative humidity between 90 and 95%, growth took place in some experiments, but usually there was a slow decline in count. Only one time was there evidence of spore formation in the instances where growth was recorded. Both total and spore counts were at a low level. These data on growth and those of Dack and Segalove *(3)* based on toxin formation are mutually confirmatory.

Growth was not observed in any experiment at 35% total moisture. Spore germination seemingly did take place since there was a slow and steady drop in spore count, which in some experiments reached to zero within as few as 5 days, although in others spores could still be detected after 4 months. In a limited series of experiments at 32% moisture results similar to those of the 35% moisture series were observed.

Similar results throughout the work for spore and vegetative cell inocula were recorded.

Spore formation by vegetative cell inocula in the absence of growth was not observed in any instance.

SUMMARY

The data from observations on growth and spore formation at moisture concentrations of from 32 to 60% indicated, for the system studied, that:

Growth and spore formation at moisture concentrations of 60% were normal.

At moisture concentrations of 50 and 45% both growth and spore formation were adversely affected, but still continued.

At moisture concentrations of 40% slight growth both with and without spore formation might be observed, but generally no growth took place.

At moisture concentrations below 40% growth and spore formation did not occur.

Spore germination, as evidenced by differences in quantitative counts at intervals of time, took place at all moisture concentrations.

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THE EFFECT OF ESSENTIAL OILS ON THE INHIBITION AND THERMAL RESISTANCE OF MICROORGANISMS IN ACID FOOD PRODUCTS[a,b]

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Because of their widespread use in many food products, the possible effect of essential oils and spices on the inhibition and thermal resistance of potential spoilage microorganisms in such foods has been of interest for many years. Although a considerable amount of work has been reported on different aspects of this problem, the results obtained do not present a clear-cut picture. The inhibitory effect of certain essential oils is well known, and it has been further shown that such action may be selective towards certain microorganisms. That this action may also be influenced by the nature of the substrate and other factors has been reported, but little information on the effect of essential oils on the thermal resistance of microorganisms associated with food spoilage has been published. In the course of work on the preservation of fruit juices and acid food products, such as that on fresh pack pickles at this laboratory, some consideration has been give to the role of essential oils in the preservation of such products.

In the present discussion some of the pertinent literature relating to this problem is reviewed, and experimental data are presented.

REVIEW OF LITERATURE

Preservative action of essential oils and spices. Guenther *(8)* has summed up our knowledge relating to the bactericidal effects of essential oils as follows: "The inhibiting and damaging effect of the oils on many life processes has been turned to our advantage in the use of these compounds as bactericidal and fungicidal agents. However, from the diversity of the compounds in essential oils, it is clear that we have to regard with suspicion any general statement on the bactericidal action of essential oils. From specific cases which have been studied, it can be concluded that the terpene derivatives, while possessing action bactericidal to certain organisms, are not able to inhibit growth in all of the numerous types of microorganisms."

Corran and Edgar *(5)* found that brown mustard and ground cloves, oils of mustard, clove, thyme, and bay leaves were effective inhibitors of the growth of *Saccharomyces rerevisiae* in beer wort. Clove and mustard oils (0.02%), and brown mustard flour (0.5%) were more effective than benzoic acid (0.06%) and sulfur dioxide (0.035%). Fabian, Krehl, and Little *(7)* reported considerable variation in the resistance of different organisms to the same spice or essential oil and of the same organism to different spices. Ground cinnamon and cloves were the only spices that exhibited inhibiting action on bacterial growth in low concentrations. In nutrient agar against most of the bacteria tested, ground peppercorn and allspice showed inhibiting action in 1% concentrations; mustard, mace, nutmeg, and ginger in 5% concentrations; and celery, in 10 and 20% concentrations. Spice oils and oil emulsions were more inhibitory than the ground spices.

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[b] Contribution No. 846 Massachusetts Agricultural Experiment Station.

Blum and Fabian *(4)* studied the effect of 32 spice essential oils and 7 components on the growth of certain organisms growing at the surface and on certain true yeasts growing beneath the surface of fermented products. The relative inhibition of the spice oils was not the same for all tests and organisms. Oil of mustard was consistently superior to the others, after which came cinnamon, cassia, and cloves. The resistance of the organisms to the action of the spices varied with the organisms and the spice. The organisms in the order of their decreasing resistance were *Acetobacter aceti*, *Saccharomyces ellipsoideus* and *cerevisiae*, and *Mycoderma vino*.

Webb and Tanner *(14)* reported that spice oils, because of greater concentrations of active principle, were more direct in disinfectant action than were whole or ground spices. Spices were effective inhibitors of yeast growth under some conditions, but yeasts could adapt themselves to low concentrations of spices and oils and in some instances appeared to be stimulated by them.

From a review of the literature, Potter *(12)* concluded that spice oils may exert a measurable influence on the keeping qualities of a product and that their efficacy as antiseptics is frequently higher than some chemical antiseptics. Kosker, Fellers, and Esselen *(11)* reviewed the literature relating to the preservative action of mustard and mustard oil. They also reported that 11 to 22 p.p.m. of mustard oil or its equivalent in ground mustard seed exerted a favorable preservative action in fresh apple cider and in grape juice.

On the other hand, James *(9)*, Webb and Tanner *(14)*, and others have pointed out that while there has been a general belief that spices possess preservative properties in foods, there is also considerable evidence that this action may, at most, be of only limited intensity. Because of the large numbers of microorganisms found on whole and ground spices the reputed preservative action of spices in foods is questionable. Today the desirability of treating whole and ground spices to reduce their microbial load is generally recognized as desirable to reduce the contamination of food products in which they are used.

Effect of essential oils and spices on thermal resistance of microorganisms. Although many investigations have been reported relating to the preservative action of essential oils and spices, relatively little work has been reported on the effect of these materials on the thermal resistance of microorganisms. From a review of available information, Baumgartner *(3)* concluded that the effect of elevated temperature on the toxic action of spices on microorganisms does not appear to have been investigated, but it would seem possible that at the temperatures used for processing canned foods, the action of these spices might cause an appreciable reduction in the heat resistance of microorganisms. He further suggested that the essential oils of spices, such as cloves and mustard, which are known to be toxic to microorganisms at normal growth temperature, may have, at processing temperatures and in low concentrations, an appreciable effect in reducing heat resistance.

Anderson, Esselen, and Fellers *(1)* found that black pepper (0.1 and 1.0%) and oil of clove (0.01 and 0.1%) had little or no effect on the thermal resistance of *Bacillus thermoacidurans* in tomato juice. Kosker, Esselen, and Fellers *(10)* reported that the presence of 10 p.p.m. of allylisothiocyanate (mustard oil) in buffer solution and in apple and grape juices caused a marked reduction in the thermal resistance of *Aspergillus niger* and *Saccharomyces ellipsoideus*, but *Bacillus thermoacidurans* was less sensitive to this compound. Oil of garlic and oil of onion had less effect on the thermal resistance of *Bacillus thermoacidurans* than did mustard oil.

Anderson *et al. (2)* studied the effect of a Kosher dill spice oil flavoring and of mustard oil on the thermal resistance of a yeast isolated from spoiled fresh pack pickles. The dill flavoring was composed of both garlic and dillweed oils combined with vegetable gum into a standard emulsion. The results of adding the dill spice oils in concentrations of 258 p.p.m. (acceptable flavoring level) and 2,580 p.p.m. and mustard oil at a level of 20 p.p.m. to a brine containing 0.5% acetic acid and 5.0% salt were evidenced by a greatly reduced thermal resistance of the test yeast.

EXPERIMENTAL

The following test organisms used in this study were obtained from the American Type Culture Collection: *Leuconostoc mesenteroides* No. 8293, *Saccharomyces cerevisiae* No. 2373, *Lactobacillus fructovorans* No. 8288, *Saccharomyces ellipsoideus* No. 4097,

Lactobacillus buchneri (lycopersici) No. 4005, and *Zygosaccharomyces globiformis* No. 10,744. Strain No. 43-P of *Bacillus coagulans (thermoacidurans)* was obtained from the National Canners Association. Organism No. 7D was a yeast which was originally isolated by Anderson *et al. (2)* from spoiled jars of fresh processed pickles. All the organisms were carried in plastic screw-capped test tubes on dextrose agar slants.

Essential or spice oils were supplied[c] as oil in water emulsions with a mixture of soluble gums as the emulsifying agent. Dill weed, celery seed, cinnamon (USP), cinnamon bark, clove (USP), garlic dill flavor, mixed pickling spice (A), mixed pickling spice (B), and Polish style garlic dill flavor were 21% essential oil emulsions, whereas onion, garlic, and mustard were only 6.25% emulsions.

Inasmuch as all test organisms grew well in regular dextrose broth (pH 7.2), this medium was selected for the initial study on the relative inhibitory effect of the various essential oils. To 10 ml. of dextrose broth, the following concentrations of all essential oils except mustard, garlic, and onion were added aseptically: 0, 21, 42, 63, 84, 105, 210, 420, 630, 840, and 1,050 p.p.m. In the latter oils, the concentrations were lowered to 0, 6, 12, 19, 25, 31, 62, 125, 187, 250, and 306 p.p.m. On the basis of available information for all the essential oils other than mustard, garlic, and onion, a normal flavoring level was considered to exist at a concentration of approximately 210 p.p.m.

One drop of an actively growing 48-hour culture of the test organism in dextrose broth was inoculated into tubes containing sterile dextrose broth and the various essential oil concentrations. Cotton plugs were used in the tubes throughout the runs in dextrose broth at pH 7.2. After incubation for 5 days at room temperature, observations of turbidity, sedimentation, or pellicle formation were made to determine the levels of essential oils that proved inhibitory to the growth of the various test organisms. At the higher spice concentrations, i.e., from 630 to 1,040 p.p.m., discretion had to be exercised to differentiate between the turbidity and sedimentation associated with microbiological growth and that imparted by the gum carriers of the essential oils. The results are listed in Table 1.

A second and similar series of experiments was made in dextrose broth adjusted to pH levels of 4.0, 4.5, or 5.0. Only in *Leuconostoc mesenteroides* was it necessary to add 0.5% yeast extract to the dextrose broth to obtain good growth in the normal incubation period of 5 days. In all cases, the pH level used was the lowest at which the test organism would readily grow. Thus *Leuconostoc mesenteroides* would grow well at pH 5.0 but not at pH 4.5, and similarly the growth of the yeast pickle spoilage organism No. 7D was good at pH 4.0 but not at pH 3.5. To prevent any change in the growing conditions of the test organisms, the suspensions used to inoculate the tubes containing the essential oils were grown in dextrose broth adjusted to the same pH as that at which the test was to be made. Inasmuch as the addition of acetic acid to dextrose broth medium resulted in the formation of a precipitate, it was necessary to filter the medium before tubing and sterilizing.

Also in view of the fact that most essential oils are somewhat volatile at room temperature, the second series of experiments was made in test tubes fitted with plastic screw caps rather than with cotton plugs. The data obtained are summarized in Table 2. A series of experiments comparing screw capped tubes to the latter revealed that in the 5-day incubation period there was a slight but consistent increased inhibitory effect from the same concentration of an essential oil in the screw-capped tubes over that in the cotton plugged tubes. In no case did this increased inhibitory effect exceed that resulting from the next higher spice oil concentration in the cotton plugged tubes.

Influence of essential oils on thermal resistance. Experiments to date have been concerned with the influence of varying concentrations of essential oils at elevated temperatures on a, thermal resistance of *Bacillus thermoacidurans* in tomato juice, and b, the thermal resistance of the yeast pickle spoilage organisms No. 7D in a brine medium (0.5% acetic acid and 5.0% salt).

In the studies of *Bacillus thermoacidurans*, spore crops were prepared according to the method employed by Anderson, Esselen, and Fellers *(1)*, which resulted in a physiological saline suspension containing 500,000 spores per ml.

A modification of the multiple tube method of Esty and Williams *(6)* was used in all studies on the influence of essential oils on the heat resistance of the test organisms.

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[c] By the Magnus, Mabee, and Reynard, Inc.

TABLE 1

Results of tests measuring the inhibitory activity of essential oils against acid food spoilage organisms in dextrose broth at pH 7.2

	Leuconostoc mesenteroides	*Lactobacillus lycopersici*	*Saccharomyces cerevisiae*	*Saccharomyces ellipsoideus*	*Zygosaccharomyces globiformis*	Yeast spoilage organism No. 7D
	P.P.M. oil required for inhibition during 5 days of incubation					
Dill weed oil	1050+	1050+	630	1050	1050+	1050+
Celery seed oil	630	840	210	420	1050+	1050+
Cinnamon oil (USP)	630	210	84	63	63	63
Cinnamon bark oil (Ceylon)	420	420	84	42	84	105
Clove oil	420	840	630	420	840	840
Garlic dill flavor	1050	1050	420	210	1050	1050+
Mixed pickling spice oils (A)	1050+	1050+	210	84	210	420
Mixed pickling spice oils (B)	1050+	1050+	630	1050+	420	1050+
Polish style garlic dill flavor	1050	420	420	210	105	840
Garlic oil	310	26	<6	<6	19	31
Onion oil	310+	260+	310+	19	26	310+
Mustard oil (artificial) USP	42	42	8.4	4.2	4.2	42

+ = growth at that concentration.

TABLE 2

Results of tests measuring the inhibitory activity of essential oils against acid food spoilage organisms in acidified dextrose broth

	Leuconostoc mesenteroides pH 5.0	*Lactobacillus fructoverans* pH 4.5	*Lactobacillus lycopersici* pH 4.5	*Bacillus thermoacidurans* pH 5.0	*Saccharomyces cerevisiae* pH 4.5	*Saccharomyces ellipsoideus* pH 4.5	*Zygosaccharomyces globiformis* pH 4.5	Yeast spoilage organism No. 7D pH 4.0
	P.P.M. oil required for inhibition during 5 days of incubation							
Dill weed oil	420	....	840+	84	63	84	42	840
Celery seed oil	....	....	....	....	42	84	....	840+
Cinnamon oil (USP)	....	21	105	63	63	42	21	210
Cinnamon bark oil (Ceylon)	210	....	210	84	84	42	63	210
Clove oil	....	....	....	....	840+	840+	....	840+
Garlic dill flavor	....	42	420	63	105	63	63	840+
Mixed pickling spice oils (A)	....	84	420	42	84	42	21	....
Mixed pickling spice oils (B)	630	....	840+	105	210	84	63	....
Polish style garlic dill flavor	420	....	630	....	42	42	42	....
Garlic oil	....	13	26	6	6	6	6	13
Onion oil	....	6	32	6	6	6	6	19
Mustard oil (artificial) USP	....	<21	<21	<21	<21	<21	<21	<21

+ = growth at that concentration.

After inoculating 50-ml. aliquots of sterile tomato juice, containing the various concentrations of the essential oils, with sufficient spore suspension to give a final concentration of 10,000 spores per ml., 2-ml. aliquots of the well-dispersed mixture of tomato juice, essential oil, and spores were transferred to standard thermal death time (TDT) tubes. The open ends of the TDT tubes were then sealed in an oxygen flame. Six TDT tubes were placed in specially constructed racks and heated in constant temperature oil bath for various time intervals allowing a lag time of 2.5 minutes as previously determined by Anderson, Esselen, and Fellers *(1)*.

The constant temperature oil bath was maintained at a temperature of 212°F. (100°C.) by means of a Beckman thermoregulator and rapid mechanical agitation of the oil. Upon completion of the heating time, the racks were removed from the oil and immersed in ice water. The TDT tubes were then removed from racks, wiped off, and their contents aseptically transferred to sterile test tubes. Because of the known inhibitory effect of tomato juice on the growth of *Bacillus thermoacidurans* in the proteose peptone acid agar (PPAA) medium suggested by Stern, Hegarty, and Williams *(13)*, 0.1- rather than 1.0-ml. aliquots were transferred to sterile Petri plates. In view of the results obtained in the inhibition studies of the effect of various essential oils on *Bacillus thermoacidurans*, the incorporation of only 0.1 ml. of tomato juice and essential oil mixture into 18 ml. of PPAA should reduce to a minimum any inhibitory effects caused by the essential oil. Eighteen ml. of sterile PPAA were then added, and the plates incubated for 96 hours at 98.6°F.(37°C.) before being counted. In this way the reduction in numbers of spores could be followed through the various heating intervals until all spores were destroyed. The results of this work are recorded in Table 3.

TABLE 3

Effect of various concentrations of essential oils in tomato juice on the thermal resistance of B. thermoacidurans at 212°F.(100°C.)

Essential oil	Concentration	Time at 212°F. to give 100% destruction
	(p.p.m.)	*(minutes)*
Control	0	12
Clove	105	11
Clove	210	11
Clove	420	8
Garlic dill flavor	105	11
Garlic dill flavor	210	11
Garlic dill flavor	420	11
Cinnamon	105	11
Cinnamon	210	10
Cinnamon	420	7
Garlic	31	8
Garlic	62	7
Garlic	125	7

Using organism No. 7D, a yeast isolated from spoiled jars of fresh processed pickles, studies were conducted to determine the effect of varying concentrations of essential oils on its thermal resistance in a brine (0.5% acetic acid and 5.0% salt—pH 2.7). To sterile 50-ml. aliquots of the brine in 8-ounce screw cap dilution bottles were added sufficient amounts of the essential oil emulsions to give the following 4 concentrations: 420, 210, 105, and 0 p.p.m. Thus, if as previously indicated, 210 p.p.m. of most essential oils approximates a normal flavoring level, the above concentrations would give results representing one-half and double the usual essential oil concentration as compared to the control containing no oil.

One-ml. aliquots of suspensions representing the 48-hour growth of organism No. 7D in dextrose broth served as inocula for the 50-ml. mixtures of pickle brine and essential oils. This resulted in a final concentration of approximately 700,000 organisms per ml. Inasmuch as the 420 p.p.m. concentration of certain essential oils had a lethal effect on

organism No. 7D, even at room temperatures, determinations of the effect of these oils at temperature of 120-135°F.(49-56.8°C.) had to be conducted with the utmost speed. Immediately after adding the inoculum to the mixture of pickle brine and essential oil and shaking thoroughly, one-ml. aliquots were transferred to sterile TDT tubes by means of sterile 10 ml. pipettes. As soon as the TDT tubes were filled, they were placed in an ice water bath, being removed only to seal their open ends in an oxygen flame. Replicates of at least three TDT tubes were then placed in racks and treated as outlined in the procedure for *Bacillus thermoacidurans*. However, after removal from the ice water, the entire contents (ca. 1 ml.) of the TDT tubes were aseptically transferred to screw-capped test tubes containing 10 ml. of dextrose broth. These tubes were then incubated for 96 hours at room temperature. The initial growth of this organism is characterized by a sedimentation which is rapidly followed by the appearance of a characteristic pellicle. These results are presented in Table 4.

DISCUSSION

From the results presented in Table 1, it is apparent that essential oils vary widely in their inhibitory action against the test organisms employed in this investigation. In dextrose broth at pH 7.2, dill weed oil was the least effective of the 12 essential oils in inhibiting the growth of the various organisms. This was followed, in order of increased effectiveness, by mixed pickling spice oils (A), garlic dill flavor, celery seed oil, mixed pickling spice oils (B), Polish style garlic dill flavor, and clove oil. The essential oils most effective at this pH level were mustard, garlic, onion, and cinnamon.

Of all test organisms, with very few exceptions, *Leuconostoc mesenteroides* was the most resistant to the higher concentrations of the essential oils. Generally speaking, in the order of their decreasing resistance to the essential oils, the organisms were *Leuconostoc mesenteroides*, *Lactobacillus lycopersici*, yeast organism No. 7D, *Zygosaccharomyces globiformis*, *Saccharomyces ellipsoideus* and *Saccharomyces cerevisiae*.

When the same essential oils were incorporated into dextrose broth at pH levels of 4.0, 4.5, or 5.0—as listed in Table 2—for the most part the inhibitory action of the essential oils was increased. Thus, in dill weed oil only 4% of the concentration necessary to inhibit the growth of *Zygosaccharomyces globiformis* in dextrose broth at pH 7.2 was required to prevent growth in the same medium at pH 4.5. Similarly, for each of the test organisms studied, lower concentrations of dill weed, celery seed, garlic dill flavor, mixed pickling spice oils (A) and (B), garlic and onion oils were adequate in preventing growth in dextrose broth at pH 4.5 than pH 7.2. more adequate in preventing growth in dextrose broth at pH 4.5 than pH 7.2.

An exception to these findings was the yeast organism No. 7D which in 3, and possibly 5 cases, required as great or greater concentrations of essential oils in dextrose broth at pH 4.0 as in the same medium at pH 7.2. This may be partially due to the fact that this organism was isolated as the causative spoilage agent in many jars of fresh processed dill pickles whose normal pH is below 4.0. Consequently, it is not unlikely that the optimum pH for its growth in dextrose broth would be closer to pH 4.0 than to 7.2, and therefore increased concentrations of certain essential oils might be required for its inhibition.

In *Saccharomyces cerevisiae*, *Saccharomyces ellipsoideus*, and yeast spoilage organism No. 7D, which were the only organisms on which com-

parative inhibitory data for clove oil were obtained at the 2 pH levels, it is of interest to note that for each of these organisms higher concentrations of clove oil are required in dextrose broth at pH 4.0 and 4.5 than at pH 7.2. Similar results were obtained with mustard oil in 4 of the 6 test organisms. The fact that all 3 organisms in the studies on clove oil, as well as 3 of the 4 organisms in the studies on mustard oil, were yeasts suggests that here again their optimum pH for growth lies below pH 7.2, and consequently higher concentrations of these oils may be required for their inhibition.

From the data in Table 3, it can be seen that at a flavoring level of 210 p.p.m. for clove, garlic dill flavor, and cinnamon oils in tomato juice, the destruction times of *Bacillus thermoacidurans* were only slightly reduced at 212°F.(100°C.). In garlic oil, which was the most effective of the essential oils in lowering the thermal resistance of *Bacillus thermoacidurans*, a concentration of 62 p.p.m. in tomato juice reduced the thermal resistance of *Bacillus thermoacidurans* by approximately 42%. The above findings are in substantial agreement with those of Anderson, Esselen, and Fellers *(1)* and Kosker, Esselen, and Fellers *(10)*.

The greatly reduced effect of the essential oils in lowering the heat resistance of *Bacillus thermoacidurans* as compared to their effect on the yeast spoilage organism No. 7D (Table 4) may be largely attributed to the relative difference in the action of these essential oils on spores as compared to vegetative cells.

TABLE 4

Effect of various concentrations of essential oils in brine on the thermal resistance of yeast organism No. 7D at 125°F.(51.8°C.) and 130°F.(54°C.)

Spice oil	Concentration	Time required for destruction at	
		125°F.	130°F.
	(p.p.m.)	*(minutes)*	*(minutes)*
Control	0	45	18
Garlic dill flavor	105	20	5
Garlic dill flavor	210	15	5
Garlic dill flavor	420	3	<1
Clove	105	15	<3
Clove	210	10	3
Clove	420	<3	< ½
Cinnamon	105	5	< ½
Cinnamon	210	< ½	< ½
Cinnamon	420	< ¼	< ¼
Garlic	31	4	1½
Garlic	62	1½	< ¼
Garlic	125	< ¼	< ¼

From the results in Table 4, it is evident that increasing the concentrations of garlic dill flavor, clove, cinnamon, and garlic oils from 105 to 210 and 420 p.p.m. markedly reduced for each essential oil the thermal resistance of the test organism No. 7D in the brine. Cinnamon and garlic oils were the most effective in this respect with concentrations of 210 and 62 p.p.m., lowering the heat resistance of organism No. 7D at 125°F. (51.8°C.) by 99 and 97% respectively.

SUMMARY AND CONCLUSIONS

Studies conducted on the minimum concentrations of various essential oils necessary to prevent the growth of *Leuconostoc mesenteroides*, *Saccharomyces cerevisiae*, *Saccharomyces ellipsoideus*, *Lactobacillus lycopersici*, *Zygosaccharomyces globiformis*, and yeast spoilage organism No. 7D in dextrose broth at pH 7.2 revealed a wide variance in the effectiveness of the different essential oils. Of the 12 essential oils considered in this investigation mustard, garlic, onion, and cinnamon had the greatest inhibitory activity.

When the pH of the dextrose broth medium was lowered to 4.0, 4.5, or 5.0, depending upon the growth requirements of the test organisms, the inhibitory action of most of the essential oils was increased. Only the yeast spoilage organism No. 7D consistently required as high or higher concentrations of the essential oils at the lower pH level.

Within practical limits concentrations of clove, cinnamon, and garlic dill flavor oils had little effect on the thermal resistance of spores of *Bacillus thermoacidurans* in tomato juice at 212°F.(100°C.). Comparable concentrations of garlic oil were much more effective.

Cinnamon and garlic oils were more effective than clove and garlic dill flavor oils in lowering the thermal resistance of the yeast spoilage organism No. 7D in a brine (0.5% acetic acid and 5.0% salt).

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ASCORBIC ACID AND SEQUESTERED COPPER[a]

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The catalysis of oxidation of ascorbic acid by copper has been well established. Typical studies are those by Joslyn and Miller *(10)*, Mawson *(16)*, Barron, De Meio, and Klemperer *(4)*, Stotz, Harrer, and King *(20)*, Thompson and Coleman *(21)*, and Weissberger and Lu Valle *(22)*. Silverblatt, Robinson, and King *(19)* have examined the kinetics of the reaction between ascorbic acid and oxygen in the presence of copper and have established it as a first order reaction.

Various mechanisms for the interaction of ascorbic acid and copper have been proposed. For example, Brown and Olson *(5)* have suggested the reduction of cupric ion to cuprous by the oxidation of ascorbic to dehydroascorbic acid. This is followed by the oxidation of the cuprous ion by atmospheric oxygen to liberate hydrogen peroxide. Presumably, other polyvalent metal ions capable of being easily oxidized or reduced could be involved in the same reaction. Joslyn and Miller *(10)* have discussed the oxidation of ascorbic acid fully.

Several studies have been made on substances which might inhibit the copper-ascorbic acid interaction. Results are summarized in Table 1.

Krishnamurthy *(12)* described oxalic acid as a powerful inhibitor of ascorbic acid oxidation, whether it be uncatalyzed, or catalyzed by copper, iron, or ascorbic acid oxidase. He suggests this activity may be due to complex formation with the catalyst, or formation of stable compounds with the substrate. Campbell and Tubb *(6)* have expanded the studies with oxalic acid and established reaction kinetics. They offer evidence that oxalic acid does not combine with ascorbic acid to form a new, more stable compound. Their work supports the concept that oxalic acid activity is due to removal of the catalytic effect of traces of heavy metals.

Jäger *(9)* showed that the tetrasodium salt of ethylene diamine tetracetic acid (Trilon B) was a good stabilizer for aqueous solutions of ascorbic acid. He found that it greatly delayed the copper catalyzed oxidation of ascorbic acid by formation of stable complexes with copper. This type of compound, Trilon, is a known powerful and stable heavy metal sequesterer under certain conditions. A publication *(2)* by Alrose Chemical Co. suggests the same compound as a stabilizer for ascorbic acid solutions at molar equivalence to copper.

Other substances have been shown to be effective inhibitors for the copper-ascorbic acid interaction. Giri *(7)* used pyrophosphates and found them effective in the pH range 5.0-7.2, using trichloroacetic acid as an

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TABLE 1

Studies on inhibitors for catalytic destruction of ascorbic acid by copper

Substance	Comments	Source
Glutathione, proteins, amino acids	Glutathione specific	Barron, Barron, and Klemperer *(3)*
Potassium cyanide, 95 : 5 CO and O_2	Effective	Barron, De Meio and Klemperer *(4)*
Aqueous extracts of liver, kidney muscle, spleen, or intestine	Presence bubbling O_2	Kellie and Zilva *(10)*
Plasma or hemolyzed erythrocytes	Effective	Kellie and Zilva *(10)*
Sodium diethyl dithiocarbamate, cystine, and cysteine	Effective	McFarlane *(16)*
Taurine and glycine	Ineffective	McFarlane *(16)*
Sodium diethyl dithiocarbamate and a, a′ dipyridyl	Effective in orange juice	McFarlane *(16)*
Oxalic acid, xanthine, uric acid, theophylline, creatinine	Effective	Krishnamurthy and Giri *(13)*
Tartaric, citric, malic, maleic, malonic, tannic, and aspartic acids	Slightly effective	Krishnamurthy and Giri *(13)*
Hydroquinone, resorcinol, salicylic acid and caffein	Ineffective	Krishnamurthy and Giri *(13)*
Oxalic acid	Powerful inhibitor	Krishnamurthy *(11)*
Pyrophosphate	Effective	Giri *(7)*
Sodium acid pyrophosphate	Effective	Krishnamurthy and Giri *(12)*
Ethylene diamine tetraacetic acid	Effective	Jäger *(9)*
Metaphosphoric acid	Effective	Joslyn and Miller *(10)*

acidifying agent. This is one of the few studies made in which a nonsequestering buffer was employed. This point will be expanded in the text Krishnamurthy and Giri *(13)* expanded this study and showed that 0.5-1.0% solutions of sodium acid pyrophosphate effectively inhibited copper or iron catalyzed destruction of ascorbic acid below pH 7.1. Pyrophosphate did not inhibit ascorbic acid oxidase.

The work of Krishnamurthy and Giri *(13)* suggests that other members of the molecularly condensed phosphate group be investigated as inhibitors for copper catalyzed oxidation of ascorbic acid. Mann and Ruchhoft *(15)* have shown condensed phosphates to be better heavy metal sequesterers than pyrophosphates.

The following study was undertaken using the condensed phosphate, sodium hexametaphosphate (SHMP).[4] It was hoped that as a result of these studies a method for preparation of fruit juices might evolve which would spare ascorbic acid during juice preparation. Some of these data of commercial interest have already been published by Morse *(18)*.

[4]Calgon, a product of Calgon, Inc., Pittsburgh, Penna.

EXPERIMENTAL METHODS

In this study the effect of copper upon ascorbic acid in pure solution was studied. Considerable effort was expended upon arriving at a non-sequestering buffer since Joslyn and Miller *(10)* showed that the buffer plays an important role in the interaction.

Ascorbic acid solutions consisting of 20 mg. of acid per 100 ml. were prepared using various buffers described below. In studies employing copper it was added from a copper sulfate solution such that the final ascorbic acid-copper mixture contained 10^{-4} moles of copper, a destructive level according to Campbell and Tubb *(6)*. Solutions were incubated in closed pint bottles held fixed in a water bath at 30°C. ± 0.1°C. Samples were removed periodically, depending upon the rate of deterioration of ascorbic acid, and titrated against 2,6-dichlorophenolindophenol in accordance with the Association of Vitamin Chemists *(1)*. All water was double distilled from an all-glass still, and pH measurements were done using a Coleman Model H_2 Meter.

The acetic acid-sodium acetate (ASA) buffer, Walpole's buffer, was used to obtain pH 3.6. However, studies were desired at 1.9 which would be comparable to 3% metaphosphoric acid (MPA) and a buffer was also required which would not interfere with the copper-ascorbic acid interaction. It was found that in order to arrive at pH 1.9 with an ASA system a starting acetic acid concentration of 58% was required. This was discarded as being impractical, especially since no data on the sequestration effects of acetic acid at this concentration are available. Accordingly a buffer which was felt would not interfere with the copper-ascorbic acid interaction was prepared using dichloroacetic acid (DCA). A 5% solution of DCA was prepared (pH 1.1) and adjusted to pH 1.9 with 1 N sodium hydroxide. This buffer was found to be stable at room temperature for over a week.

Sodium hexametaphosphate (SHMP) was used at molar equivalence to copper at 10^{-4} moles and in excess at 10^{-2} moles. It was also used as a 1.76% solution, since analysis shows metaphosphoric acid (MPA) ordinarily used for ascorbic acid stability contains this amount of sodium metaphosphate. Ethylene diamine tetra acetic acid* (EDTA) at 10^{-2} moles was also compared with 3% MPA. Finally, McIlvaine's phosphate-citrate buffer (PC) described by Hodgman *(17)* was used at the lowest obtainable pH, 2.4. A Waring Blendor was found to be of great assistance in preparing solutions, especially SHMP and MPA.

RESULTS

The first study in this series was made in an effort to observe the stability of ascorbic acid in unbuffered solutions, in the presence and absence of copper. Titration was done in the presence of ASA at pH 3.65. The ascorbic acid solution used in these studies showed a pH of 3.75. Results are shown in Figure 1. To check interference by copper with the dye 25 ml. of copper stock solution was titrated against the dye, but no dye reduction was observed. Next a comparison was made of the stability of ascorbic acid, and ascorbic acid plus copper when both are dissolved in 3% MPA. The results of this study are also shown in Figure 1. Even in the presence of MPA copper is seen to have a deleterious effect upon ascorbic acid stability.

MPA was compared with a buffer known to be a poorer copper sequesterer, ASA. However, since the acetate system could not be adjusted to pH 1.9, the 3% MPA was adjusted to 3.6 using 5% sodium hydroxide. Results of this study are shown in Figure 2. MPA loses much of its protective action for ascorbic acid at this pH, but is still observably more effective than an acetate system.

The pure compound MPA is a very viscous material which is extremely difficult to handle in the laboratory, yet the material used in these ear-

*Sequestrene, from Alrose Chemical Co., Providence, R. I.

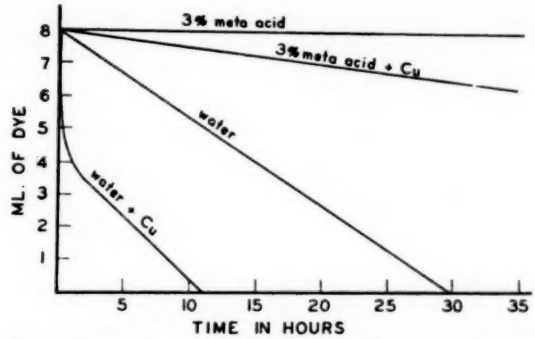

Figure 1. Rate of loss of ascorbic acid in water and 3% metaphosphoric acid in presence and absence of copper.

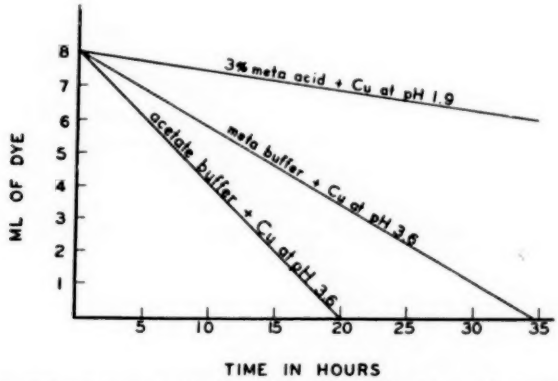

Figure 2. Rate of loss of ascorbic acid in metaphosphate and acetate buffers at pH 3.6 in the presence of copper.

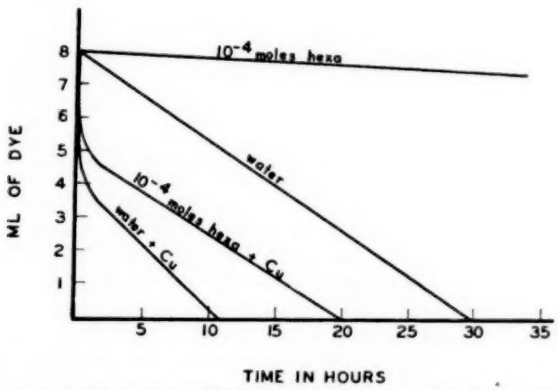

Figure 3. Rate of loss of ascorbic acid in water and 10^{-4} moles sodium hexametaphosphate in presence and absence of copper.

lier studies marked, "Metaphosphoric Acid, CP," was prepared as pellets which were extremely easy to handle and possessed a low degree of hygroscopicity. To explain this discrepancy an analysis was performed which revealed the following composition:

P_2O_5.....70.48% Na_2O.....17.82%

By calculation this indicates a composition of 58.61% sodium metaphosphate and 33.46% metaphosphoric acid. It is assumed that the remaining

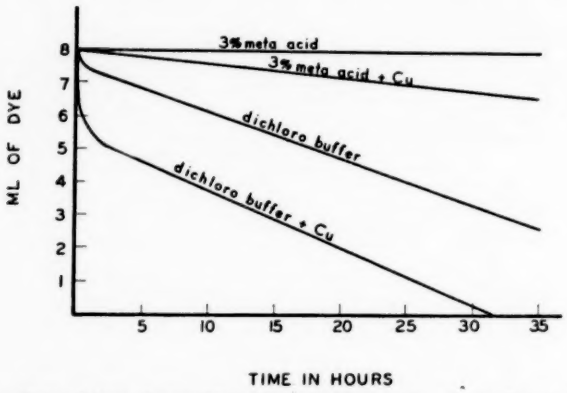

Figure 4. Rate of loss of ascorbic acid in dichloroacetic acid buffer and 3% metaphosphoric acid in the presence and absence of copper.

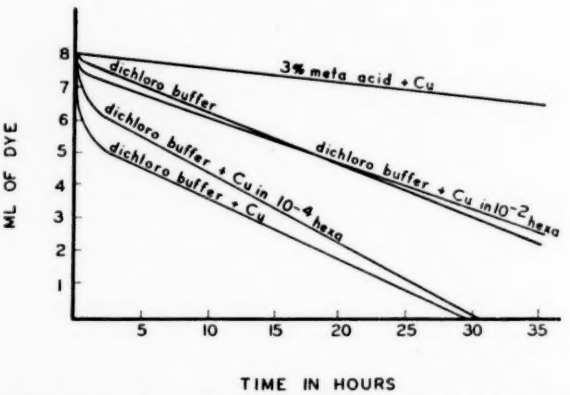
3% meta acid + Cu
dichloro buffer
dichloro buffer + Cu in 10-2 hexa
dichloro buffer + Cu in 10-4 hexa
dichloro buffer + Cu
ML OF DYE
TIME IN HOURS

Figure 5. Rate of loss of ascorbic acid in dichloroacetic acid buffer containing 10^{-2} and 10^{-4} moles of sodium hexametaphosphate in the presence of copper.

7.93% is water. The label showed no indication of sodium metaphosphate content. Since this analysis, material from another supplier has been received showing the above analysis on the label. The sodium salt is added to facilitate handling, but cognizance should be taken of this composition

for studies of this type, also its ready hydrolysis to less effective orthophosphoric acid, especially in higher pH ranges.

SHMP at 10^{-4} moles was compared with distilled water solutions of ascorbic acid in the presence and absence of copper, all at pH 3.70. Results are shown in Figure 3. In the presence of copper SHMP offers little protection at this level.

Three percent MPA was compared with DCA in the presence and absence of copper. Results are shown in Figure 4. DCA buffer offers little protection; the meta acid even with added copper is considerably more protective than DCA.

SHMP was added to DCA buffer at 10^{-4} and 10^{-2} moles and these mixtures were compared with MPA, shown in Figure 5. From this study it is seen that SHMP at the levels studied is not equivalent in protective action to MPA. While SHMP offers no protection at 10^{-4} moles, at 10^{-2} moles it nullified the effect of copper on ascorbic acid in DCA buffer.

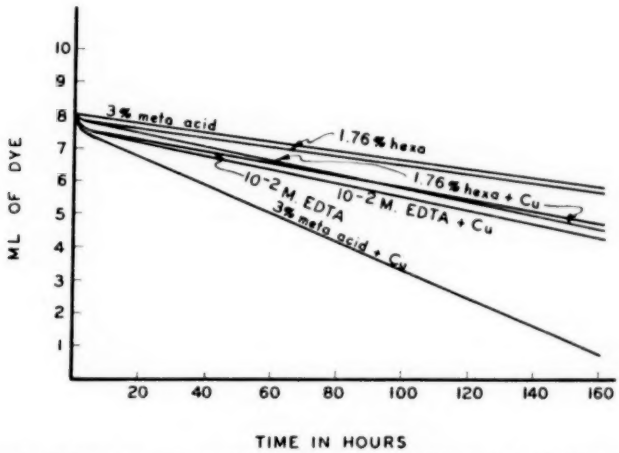

Figure 6. Rate of loss of ascorbic acid in 3% metaphosphoric acid (meta), 1.76% sodium hexametaphosphate (hexa) and ethylene diamine tetraacetic acid (EDTA) at 10^{-2} moles at pH 1.9 in the presence and absence of copper.

In the next study an attempt was made to determine the effective portion of MPA as shown by previous analysis, see Figure 6. Accordingly, a solution of SHMP was prepared such that it was equivalent to the sodium metaphosphate content of MPA. This solution was then acidified to pH 1.9, using DCA, and compared with 3% MPA and 10^{-2} molar EDTA made to pH 1.9 with DCA.

Finally, a comparison was made between DCA and PC buffers. In this study pH 2.45 was employed because of the limitations of PC buffer. Results are shown in Figure 7. MPA offers far more protection than either of the other two buffers. But PC offers slightly better protection than DCA.

DISCUSSION

The stability of ascorbic acid is seen to be low in double distilled water, and in the presence of 10^{-4} moles of copper it is virtually transitory. Upon mixing with copper about half of the titratable acid disappears and after 10 hours all of the ascorbic acid disappears. As is well known, in the presence of metaphosphoric acid (MPA) ascorbic acid is quite stable even in the presence of copper. From this study it is apparent that when MPA is present, ascorbic acid is more stable in the presence of copper than it is in double distilled water even in the absence of copper.

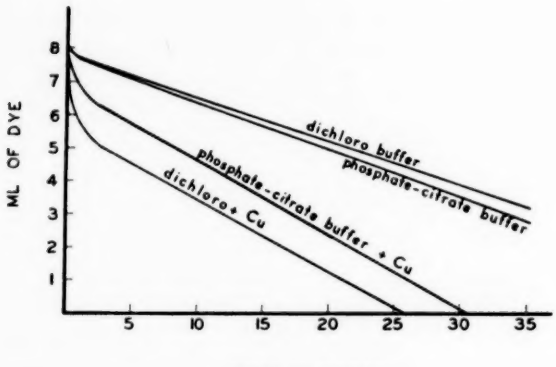

Figure 7. Rate of loss of ascorbic acid in dichloroacetic acid and phosphate-citrate buffers at pH 2.45 in the presence and absence of copper.

When MPA is compared with acetate buffer at pH 3.7, the lowest obtainable with acetate systems, metaphosphoric shows more protection than acetate but it is considerably poorer at this pH than 1.9, the pH used above, see Figures 1 and 2.

When SHMP was employed in equimolar concentration to the copper present it did not provide protection and was little improvement over plain water. However, at this concentration SHMP in the absence of copper effectively stabilized ascorbic acid almost as well as 3% MPA.

The suitability of the system dichloroacetic acid-sodium dichloroacetate (DCA) was investigated. In the next study DCA was compared with MPA and was found to offer little protection to ascorbic acid against the deleterious effects of copper. DCA buffer offers the least protection of the buffers investigated.

In the next study DCA buffer with SHMP added at 10^{-2} and 10^{-4} moles was compared with 3% MPA. SHMP at the higher concentration nullified the effect of added copper, but this concentration was still not sufficient to equal the effect of MPA. At the lower concentration SHMP offered little additional protection.

In an attempt to match the effect of MPA, DCA buffer was reinforced with SHMP such that it was equivalent in sodium metaphosphate content to MPA according to analysis. From this study SHMP at this level is

shown to be equally effective to MPA, which is not surprising considering the large excess of sequestering power present. EDTA even at the low concentration employed is seen to be an excellent protector, and there is little difference in the stability of ascorbic acid with or without copper. This compound offers the additional advantage of being quite stable to hydrolysis.

In the final study in this series, DCA, a poor sequesterer, is compared with the buffer often used in studies of this type, phosphate-citrate (PC). DCA is shown to offer less protection than PC which was expected since citric acid and citrates are known to be good sequesterers. This raises the question of buffer interference in other studies. In observing the effect of copper and other heavy metals upon a reaction, account must be made for reaction between copper and the buffer employed.

SUMMARY AND CONCLUSIONS

The effectiveness of sodium hexametaphosphate as an anti-catalyst in the copper destruction of ascorbic acid has been investigated. McIlvaine's phosphate-citrate buffer is shown to interfere with the ascorbic acid-copper interaction probably by sequestration of copper by the citrate ion. Since the interference by buffers has been shown to be of importance in these studies, a dichloroacetic acid buffer offering minimal interference has been suggested. SHMP in the presence of an equimolar concentration of copper does not prevent ascorbic acid degradation. However, at concentrations equivalent to the sodium metaphosphate content of metaphosphoric acid of commerce it is equally effective when acidified with dichloroacetic acid.

Acknowledgment

Appreciation is expressed to Mr. Forrest Barker and Dr. Peter G. Arvan of the Phosphate Division Laboratories of Monsanto Chemical Co. for their assistance and advice during this study. Gratitude is also expressed to Mr. Kenneth Christensen for his assistance with the drawings.

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THE PECTOLYTIC ACTIVITY OF SPECIES OF THE GENUS *BACILLUS*: QUALITATIVE STUDIES WITH *BACILLUS SUBTILIS* AND *BACILLUS PUMILUS* IN RELATION TO THE SOFTENING OF OLIVES AND PICKLES[a,b]

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(Manuscript received June 12, 1952)

The problem of cucumber softening was investigated as early as 1899 by Aderhold *(1)* and, in the intervening years, has been the subject of a number of independent studies. Kossowicz *(15)* probably was the first to describe bacteria of the genus *Bacillus* as the cause of the softening of pickles for, although Aderhold *(1)* worked with similar spore-forming organisms, he reported that *Bacterium coli* caused the softening. The work of Kossowicz was confirmed and extended—first by Rahn *(24)*; later by Le Fevre *(17)* and Joslyn *(12)*; and most recently by Fabian and co-workers *(7, 8)*. From the results of these investigations the general conclusions have been drawn that bacteria are involved in the softening of pickles and that members of the genus *Bacillus* are the principal agents of such spoilage, acting through the pectolytic enzymes which they produce.

It was not possible to accept these conclusions without some reservations. Therefore, it was decided to reinvestigate the pectolytic activity of species of the genus *Bacillus*. It was thought that more convincing proof of the role of these bacteria in the softening of pickles and olives could be obtained. Also, it has been known for many years that microorganisms other than species of *Bacillus* cause softening of fruit and vegetable tissues. Some of these are plant parasites. Others, such as the retting organisms, are saprophytes. The pectolytic enzymes produced by some of these organisms have been investigated *(6, 9, 16, 18, 20, 21, 22, 32)*.

EXPERIMENTAL METHODS

Isolation and identification of cultures. A differential medium in which agar was replaced by sodium ammonium pectate as suggested by Baier and Manchester *(2)* was used for isolation of the cultures. The medium had the following composition:

Sodium ammonium pectate	45 g.
Bacto-peptone	5 g.
Bacto-beef extract	3 g.
Calgon (food grade)	2.5 g.
Sodium propionate	1 g.
Water	1,000 ml.

[a] Presented at the Twelfth Annual Meeting of the IFT, Grand Rapids, Michigan, June 10, 1952.

[b] Support of this study with funds from Grants-in-aid to the University of California by the California Olive Association, the State of California Olive Advisory Board, and the American Spice Trade Association is gratefully acknowledged.

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The pH of the above mixture was adjusted to 7.7 with 1 *N* NaOH. When the medium was sterilized at 15 p.s.i.g. for 15 to 20 minutes the final pH value was about 6.5. This medium, used in petri dishes, was a good differential medium for the isolation of aerobic, pectolytic bacteria for, although it supported the growth of a variety of other bacteria, only those organisms which were pectolytic caused liquefaction of the pectate gel surrounding the individual colony. A plate of the medium, inoculated with such an organism was completely liquefied in 4 to 6 days.

The bacteria to be studied were isolated from the brines of active and dormant fermentations of salt stock and dill pickles, olives stored for ripe pickling, spanish type olives, and sauerkraut. Brines from normal and softened pickles and olives and normal sauerkraut were sampled. Most of the samples were from commercial fermentations. The desired cultures were picked from plates of the pectate medium which had been streaked with swab samples of the brines and incubated at 30°C.(86°F.). Although, in most cases, softening of the pectate gel was obvious after 24 hours, sometimes it was necessary to incubate the plates for 48 hours before picking the cultures.

Pure cultures of the pectate liquefying bacteria were obtained by restreaking the first isolates 5 consecutive times on nutrient agar. The purified cultures then were tested again to determine whether they still liquefied the pectate medium. The cultures finally chosen for study were identified according to the methods suggested by Smith, Gordon, and Clark *(28)*.

Preparation of enzyme solutions. Crude enzyme preparations were obtained by use of the beet molasses broth medium, prepared as directed by Fabian and Johnson *(7)*, or nutrient broth which contained 1% sodium ammonium pectate or 0.5% galacturonic acid.

To obtain the crude enzyme solutions 2 liters of the desired medium were inoculated with the particular culture to be studied. After incubation for 12 days at 30°C.(86°F.) the majority of the cells and other debris were removed by filtration. The clear filtrate which contained the enzymes was preserved with 0.2% sodium benzoate and a few drops of toluene.

Methods for characterizing the enzymes. Considerable difficulty was experienced in the attempts to characterize the enzymes produced by the bacilli. The methods described by Reid *(25)* for the semi-quantitative estimation of the pectin esterase and polygalacturonase produced by microorganisms did not give satisfactory results in the hands of the authors. It also was found to be impossible to use the Willstatter-Schudel hypoiodite method as modified by Jansen and MacDonnell *(11)* for determination of the hydrolysis of glycosidic bonds of pectin because the cultures tested produced such small quantities of the polygalacturonase-like enzyme. Therefore, since the main objective of this study involved determination of the role of the bacilli in the softening of cucumber pickles and olives, it was decided to more completely characterize the enzymes by quantitative means in a separate study and to resort to the use of qualitative methods for study of the enzyme activity as it would be related to softening of these pickled foods. Consequently, the effects of temperature, pH, and other factors on the activity of the crude enzyme preparations were determined by measurement of changes in the viscosity of pectinous substrates induced by the enzymes.

The decreases in viscosity of the pectate solutions were measured in an Ostwald viscosimeter maintained at a constant temperature in an oil bath. The rate of flow of distilled water through this viscosimeter was 10 seconds when measured at 21.5°C. (70.7°F.). The pectate solutions were prepared from commercial sodium ammonium pectate as suggested by Ibarra *(10)*. Conditions such as the volume of pectate solution, volume of enzyme preparation, the pH of the reaction mixture, reaction time, and the temperature at which the determination was made varied according to the experiment.

Before making an activity measurement, the pectate solution, enzyme preparation, and the viscosimeter, respectively, were brought simultaneously to the temperature at which the experiment was to be conducted. Then a suitable volume of pectate solution followed by a smaller amount of enzyme preparation was pipetted into a 50-ml. glass-stoppered Erlenmeyer flask. The exact time of mixing of these 2 solutions was recorded. After mixing thoroughly, 10 ml. of the reaction mixture was transferred into the viscosimeter. The time of flow through the viscosimeter was measured every 5 minutes.

Because heating affected the viscosity, the initial time of flow for every determination was obtained by plotting the flow values, measured at 5-minute intervals, on a graph. Then, by extrapolation of the curve, the viscosity value at the time of mixing was obtained. This value is referred to as the "initial time of flow" and represents

the time in seconds required for the reaction mixture to flow through the viscosimeter at the start of the experiment.

The activity of the enzyme was expressed as a percent loss in relative viscosity according to the formula:

$$\text{Percent loss in viscosity} = \frac{(B-A)\times 100}{B}$$

where

B = initial time of flow (in seconds)
A = final time of flow (in seconds).

In cases where the crude enzymes were prepared from media to which salt had been added, the filtrates were dialyzed against distilled water for 8 hours before the enzyme activities were measured.

The softening of cucumber and olive tissues. Fresh, salt stock, and finished sweet cucumber pickles as well as fresh and "ripe process" olives were used to test the activity of the enzymes. The fresh produce was tested without pretreatment but the salt stock, finished sweet pickles, and canned "ripe process" olives were freshened for 24 hours in several changes of water before being submerged in the crude enzyme solutions.

Ability of the enzymes to soften these fresh and desalted whole fruits was tested with preparations adjusted to pH values ranging from 4.8 to 8.0, preserved with toluene, and incubated at room temperature. Softening was judged at daily intervals by sensory means. Care was taken to insure that extraneous microorganisms did not grow in the enzyme preparations or on the submerged fruits. Controls for this purpose consisted of samples of enzyme preparation heated for 10 minutes at 80°C.(176°F.) before adding the fruits and additional toluene used for preservation of the mixtures.

RESULTS

Identification of the bacteria. The cultures which softened the sodium ammonium pectate medium were found to be members of the genus *Bacillus*. All 43 cultures were catalase-positive, mesophilic, aerobic, sporulating, gram-positive rods which acidified various substrates without visible signs of gas formation. Spores of all cultures were ellipsoidal to cylindrical in shape and were found in a central position in the sporangia which were not distinctly bulged. The individual rods were less than 0.9 of a micron in diameter. The cultures fell into two groups. Twenty-one of them were similar to the species *Bacillus subtilis*. The other 22 isolates resembled the species *Bacillus pumilus*. The differential characteristics which permitted this species allocation are shown in Table 1. On the basis of these results

TABLE 1

Differential characteristics of the bacteria

Character[a]	Group I 21 cultures	Group II 22 cultures
	Number of positive cultures	
Growth at pH 6.0	21	22
	pH range 4.0 to 9.0[b]	pH range 4.5 to 8.5[c]
V.-P. reaction (acetoin from glucose)	21	22
Gelatin hydrolysis	21	22
Acid from arabinose and xylose	21	22
Starch hydrolysis	21	0
Black pigment formation	0	0
Nitrate reduction	21	0
Species allocation	***B. subtilis***	*B. pumilus*

[a] According to the methods of Smith, Gordon, and Clark (27).
[b] pH range for growth of cultures.
[c] pH range for growth of cultures.

there was perfect agreement with the previously described characteristics which were used by Smith, Gordon, and Clark *(28)* to identify the 2 species.

The resistance of B. subtilis and B. pumilus to sodium chloride and lactic acid. The tolerance of the 2 species to salt and acid was investigated according to the method of Fabian and Johnson *(7)*. Day old cultures of *B. subtilis* and *B. pumilus* were inoculated into the molasses broth containing 5, 7.5, and 10% sodium chloride, respectively (g. per 100 ml. of sample). The cultures grew and produced turbidity in the presence of 5% salt within 24 hours. In the 7.5% salt medium turbidity was observed only after 48 hours of incubation and in the medium which contained 10% salt growth was not evident until 8 or 10 days after inoculation. The cultures could be trained to tolerate 11% salt but all attempts to train them to tolerate more salt were unsuccessful. After training, the cultures of *B. subtilis* produced turbidity in the presence of 11% salt in 4 days whereas the cultures of *B. pumilus* required 6 days. The cultures of both species could grow in the presence of 0.25% lactic acid (g. per 100 ml. as lactic) contained in the molasses broth but none of the cultures could be trained to grow in the presence of higher concentrations of the acid.

Softening of cucumbers and olives by bacterial enzyme preparations. Ability of the bacterial enzymes to soften cucumbers and olives was tested according to the method already described. Results obtained with crude enzyme preparations of *B. subtilis* No. 26 and *B. pumilus* No. 38 tested with desalted cucumbers are shown in Table 2. It will be noted that the enzymes softened the cucumbers only when the preparations were adjusted to nearly neutral or alkaline pH values. Furthermore, it is to be observed that *B. subtilis* could elaborate the pectolytic enzymes in the absence of added pectic substances whereas the culture of *B. pumilus* produced the enzymes only when grown in the pectate broth.

TABLE 2

Softening of desalted cucumbers by crude bacterial enzyme preparations

pH of enzyme preparation	Crude enzyme produced by		
	B. subtilis No. 26	*B. pumilus* No. 38	*A. aerogenes* No. 6[b]
Enzymes produced in beet molasses broth			
4.5	—[1]	—	—
5.5	—	—	—
6.5	+[2]	—	—
8.0	+	—	
Enzymes produced in nutrient broth with sodium ammonium pectate			
4.5	—	—	—
5.5	—	—	—
6.5	+	+	—
8.0	+	+	—

[b] Control culture.
[1] — indicates no softening. If the cucumbers remained firm after being submerged in the enzyme solution for 4 days at room temperature it was concluded that the preparation had no pectolytic activity.
[2] + indicates characteristic softening known as slipperiness.

On further investigation it was found that the enzymes of *B. subtilis* No. 26 did not soften pickles or olives at pH values below 5.7 and the en-

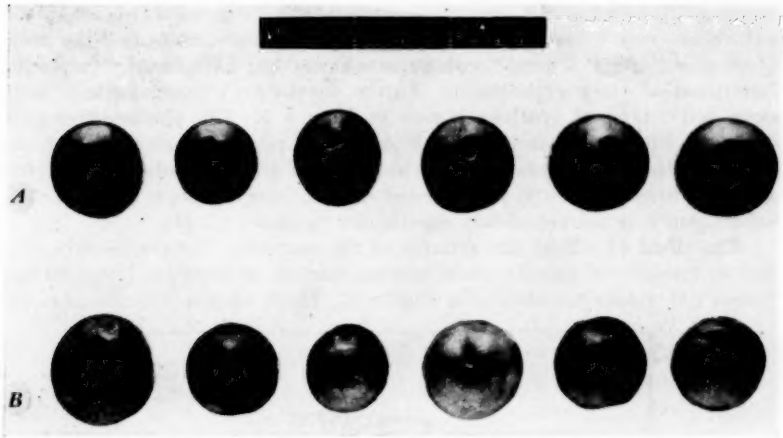

Figure 1. Typical examples of "soft stem end" spoilage of olives.
A. Normal Sevillano variety olives from storage brine.
B. Various stages of soft stem end of Sevillano olives from abnormal storage brine.

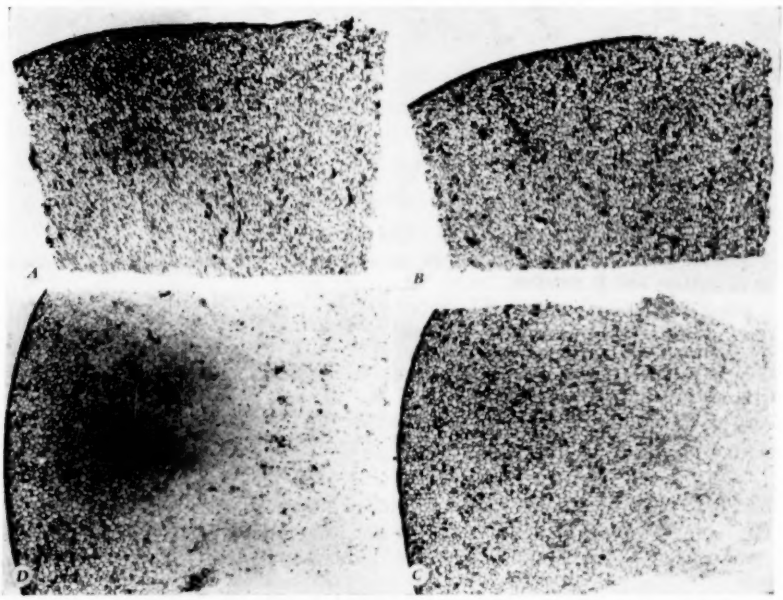

Figure 2. Photomicrographs of radial sections of normal and softened Sevillano variety olives to show probable destruction of pectinous substances. A. Normal fresh green olive section. B. Normal canned California ripe olive stained with ruthenium red. C. "Soft stem end" olive section stained with ruthenium red. Note incomplete staining of area farthest from skin. D. "Soft stem end" olive section stained with ruthenium red after treatment with Sweitzer's reagent to remove cellulose. Note large area not stained by ruthenium red.

zymes of *B. pumilus* No. 38 were incapable of causing softening when the pH values were below 6.4. The enzymes of both cultures caused the same types of softening. Cucumbers became slippery but never mushy under the conditions of these experiments. Olives developed a characteristic "soft stem end" type of spoilage shown in Figure 1. The photomicrographs shown in Figure 2 indicate that the softening probably resulted from the decomposition of pectinous substances. It should be remembered that even though ruthenium red is widely used as a differential stain for pectinous substances it is not completely specific for pectins *(13, 14)*.

The effect of pH on the activity of the enzymes. Data concerning the loss in viscosity of pectate-crude enzyme reaction mixtures adjusted to different pH values are shown in Figure 3. These results indicate that the

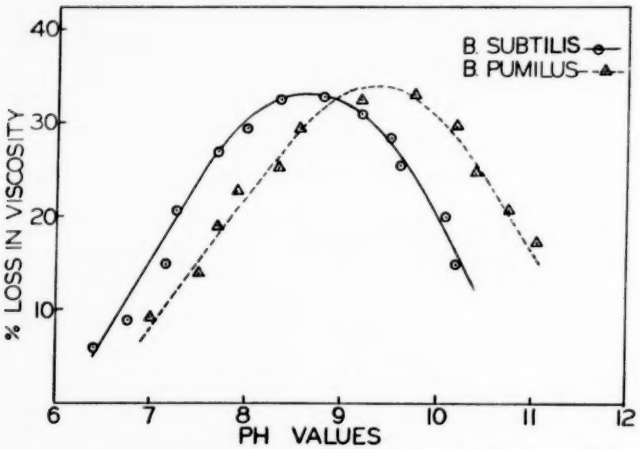

Figure 3. The effect of pH on the activity of the pectinolytic enzymes produced by B. subtilis and B. pumilus.

Conditions of experiment:
Volume of pectate solution = 20 ml.
Volume of enzyme preparation = 5 ml.
Temperature of determination = 21.5°C.(70.7°F.).
Reaction time = 30 minutes.
Viscosity reading of 40 corresponds to relative viscosity of ± 4.
Nutrient broth with 1% sodium ammonium pectate used for production of enzyme preparations.

enzymes of *B. subtilis* No. 26 are most active at about pH 8.5 whereas those of *B. pumilus* No. 38 are most active at about pH 9.4. The observations also confirm those made with cucumbers and olives that the crude enzyme preparations have very little activity below pH 6.0.

Effect of temperature on the activity of the enzymes. Determination of the decrease in viscosity is not a very satisfactory method for measuring the effect of temperature on enzyme activity because the viscosity of a pectate solution changes with temperature (see Kertesz, *14*). Nevertheless, the effect of temperature on the activity of the enzymes produced by *B. subtilis* was investigated by this method. The data obtained are shown

in Figure 4. Under these experimental conditions, with a reaction time of 30 minutes, the optimum temperature for activity was about 45°C.(113°F.). The crude preparation was completely inactivated when heated to 65°C. (149°F.) for 30 minutes.

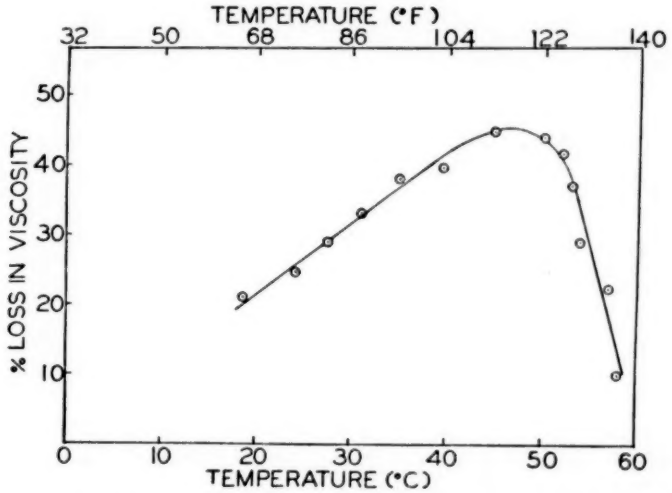

Figure 4. The effect of temperature on the activity of the pectinolytic enzymes produced by B. subtilis.

Conditions of experiment:
- **Volume of pectate solution = 30 ml.**
- **Volume of enzyme preparation = 5 ml.**
- **pH of reaction mixture = 8.0.**
- **Reaction time = 30 minutes.**
- **Nutrient broth with 1% sodium ammonium pectate used for production of enzyme preparation by B. subtilis.**

Stability of the enzymes produced by B. subtilis. Portions of preserved crude enzyme preparations of *B. subtilis* with the same initial enzyme concentration but with pH values of 4.5, 6.5, and 8.5, respectively, were stored at room temperature and analyzed for losses in activities after 3, 5, and 7 days of incubation. Before the actual viscosity measurements were made the reactions of the different enzyme solutions were adjusted to pH 8.0 and diluted so that when 5 ml. of enzyme solution was mixed with 20 ml. of pectate solution, the decrease in viscosity was less than 15%. The initial relative viscosity of the reaction mixture was about 5 at 32°C.(89.6°F.). The results of this experiment are shown in Figure 5. It is obvious that the enzymes of *B. subtilis* are inactivated rapidly in acid solution but are appreciably more stable under alkaline conditions. The sample with an initial pH value of 4.5 lost about 96% of its activity within 3 days whereas the more alkaline samples displayed considerably more stability. After 3 days the sample with a pH of 6.5 had lost 39% of its activity and the preparation with an initial pH of 8.5 had lost only 28.5% of its activity.

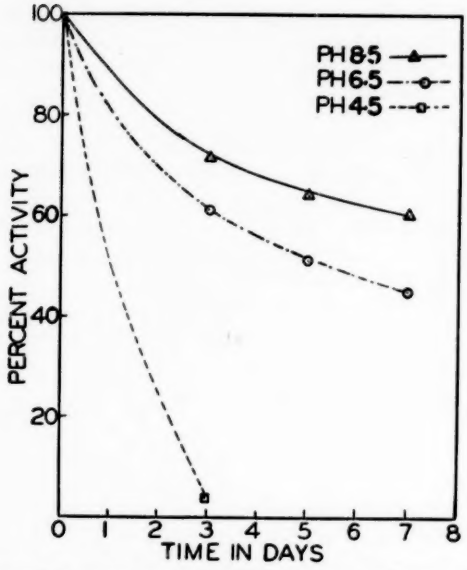

Figure 5. The effect of pH on the stability of the pectinolytic enzymes of B. subtilis stored at 23°C.(73.4°F.).

After 7 days of storage of the samples at pH 6.5 and 8.5 the losses in activity were 55% and 40%, respectively.

The effect of salt on enzyme production. The influence of salt on enzyme production by *B. subtilis* No. 26 and *B. pumilus* No. 38 was tested in a nutrient broth medium containing 0.5% galacturonic acid. The salt concentrations ranged from 1 to 10 g. per 100 ml. of this medium. The conditions under which the tests were made together with the results are shown in Table 3. Under the conditions of this experiment the culture of *B. subtilis* was capable of producing the softening enzymes in the presence of salt concentrations as high as 8 g. per 100 ml. while *B. pumilus* could produce the enzymes in the presence of 6 g. salt per 100 ml. These cultures had not been trained to tolerate large quantities of salt. Therefore, it might be possible on longer incubation of trained cultures that production of enzymes could be demonstrated in the presence of higher concentrations of salt.

The effect of pH on enzyme production. The effect of pH on enzyme production by *B. subtilis* No. 26 was tested in a nutrient broth, sodium ammonium pectate medium adjusted to pH values ranging between 4.8 and 8.7. The most attention was given to pH values between 6.0 and 8.7 because it had already been determined that the enzymes rapidly lost their activity under more acid conditions. According to the data shown in Table 5 it is apparent that enzyme production was about equal at pH values between 6.0 and 7.5 but that no production was detected at pH 4.8. However, it is to be stressed that no control of the variation in numbers of

TABLE 3

The effect of salt on softening enzyme production by B. subtilis and B. pumilus [k]

Percent salt in medium [l]	Initial time of flow [m] (B)	Final time of flow [m] (A)	Percent loss in viscosity
Enzymes produced by *B. subtilis*			
0	82.6	61.0	26.1
1	82.3	58.9	28.4
2	83.0	63.4	24.0
3	81.8	55.0	32.5
4	82.4	65.0	21.1
5	82.2	67.6	17.7
6	82.6	74.3	10.1
7	82.0	75.0	8.5
8	81.2	77.5	4.6
9	82.2	82.2	0
10	82.8	82.8	0
Enzymes produced by *B. pumilus*			
0	60.4	55.3	8.5
1	60.8	40.4	32.8
2	61.5	52.7	14.3
3	60.2	56.5	6.2
4	60.5	60.8	2.7
5	61.8	60.3	2.4
6	62.2	58.2	6.4
7	62.6	62.6	0
8	62.3	62.3	0

[k] Conditions of experiment: Nutrient broth plus 0.5% galacturonic acid and varying concentrations of salt used for enzyme production. pH of medium 6.5. Cultures incubated at 30°C.(86°F.) for 5 days. Dilution during dialysis = 25 ml. culture fluid diluted to 35 ml. Reaction mixture = 20 ml. pectate and 5 ml. enzyme preparation. Temperature of determination = 32°C.(89.6°F.). pH of determinations = 8.0. Reaction time = 30 minutes.

[l] As grams per 100 ml.

[m] Time of flow in seconds through the Ostwald viscosimeter.

TABLE 4

The effect of pH on production of pectolytic enzymes by B. subtilis [n]

pH of medium	Initial time of flow [o] (B)	Final time of flow [o] (A)	Percent loss in viscosity
4.8	64.3	64.3	0
6.0	65.5	32.8	49.9
6.4	66.3	32.2	51.4
6.8	63.4	33.3	47.5
7.5	64.8	32.0	50.6
8.2	66.8	36.2	35.3
8.7	66.4	60.6	8.7

[n] Conditions of experiment: Test medium consisted of nutrient broth with 1% sodium ammonium pectate, 0.5% glucose, and 1.2% K_2HPO_4 adjusted to different pH values. Cultures incubated at 30°C.(86°F.) for 5 days. Dilution for determination = 20 ml. made to 100 ml. Reaction mixture = 20 ml. of pectate solution and 5 ml. of enzyme preparation. Temperature of viscosity determinations = 32°C.(89.6°F.). pH of determinations = 8.5.

[o] See Table 3.

bacteria at the different pH levels or the inactivation of the enzymes during incubation was attempted in this experiment.

DISCUSSION

As stated, several unsuccessful attempts were made to quantitatively characterize the softening enzymes of *B. subtilis* and *B. pumilus*. How-

ever, on the basis of the qualitative viscosity experiments already described it seems reasonable to believe that at least one component of the softening enzyme complex of these bacteria is a polygalacturonase-like enzyme. This belief is strengthened by the fact that the enzymes of the 2 species caused some diminution in the calcium precipitate when the cultures were grown in nutrient broth plus 2% pectin NF at initial pH values of 6.6 and 8.6 at 30°C.(86°F.) for 5 days. Furthermore, when the cultures were grown in nutrient broth plus 0.5% pectic acid for 5 days at 30°C.(86°F.) gel formation could not be demonstrated after the addition of required amounts of molar $CaCl_2$, normal HCL, and 95% ethanol to different portions of the medium although good gel formation was observed in the medium in which *A. aerogenes* had been grown as well as in the uninoculated, sterile control. These observations all suggest the presence of a polygalacturonase-like enzyme.

The enzymes produced by *B. subtilis* and *B. pumilus* were similar in certain respects to the polygalacturonase of *Erwinia (Bacterium) aroideae* which was described recently by Wood *(32)*. The enzymes produced by all 3 species were most active in the alkaline range and were rapidly inactivated in acid surroundings. This appears to be characteristic of the polygalacturonase-like enzymes produced by bacteria [also see Fernando *(9)*, Oxford *(20)*, and Kraght and Starr *(16)*] whereas the reverse is true of the polygalacturonase produced by fungi according to the work of Ehrlich *(6)*, Waksman and Allen *(32)*, Phaff *(22)*, Luh and Phaff *(18)*, Pavgi, Pithawala, Savur, and Sreenivasan *(21)*, and others.

Fabian and his co-workers *(7, 8)* adhere to the belief that *B. mesentericus fuscus* and *B. vulgatus*[p] produce enzymes which can soften cucumber pickles. On the other hand, Bell, Etchells, and Jones *(3)* have presented evidence which indicates that the enzymes capable of softening commercial cucumber salt stock are of fungal origin. Because the pectolytic enzymes produced by *B. subtilis* and *B. pumilus* are inactivated rapidly at pH values below 5.7 and 6.4, respectively, it appears unlikely that common pectolytic bacteria of the genus *Bacillus*, regardless of origin (soil, water, spices, equipment, cucumbers, olives, etc.) are involved in the softening of normal salt stock and fermented dill pickles or storage, sicilian-type and spanish-type olives,[q] all of which undergo an acid fermentation.

On the other hand, the 2 pectolytic species of *Bacillus* studied, as well as other bacteria, most probably are involved in the softening of olives being processed for the canned California ripe and green-ripe olives of commerce.[q] In these 2 processes the acidities of the fresh or storage fruits are neutralized rapidly by the sodium hydroxide used and, from that point on, always have pH values in the neutral to alkaline range where the known fungal enzymes apparently are not active. These bacteria also probably are involved in the softening of spanish-type green olives. In their preparation for fermentation sodium hydroxide is used to destroy part of the objectionable bitterness normal to the fresh olives. During the washing

[p] See Smith, Gordon, and Clark *(28)* and Smith *(27)* for the synonomy of the 2 species.

[q] Consult Cruess *(4, 5)*, Vaughn *(29, 30)*, and Vaughn, Douglas, and Gililland *(31)* for the technical details of olive pickling.

operations following the lye treatment the pH values frequently remain in the alkaline range for several days. Then, if the normal acid fermentation does not start, the olives may remain at or near neutrality for several weeks or months and afford ample time for the bacteria to elaborate softening enzymes in favorable surroundings.

SUMMARY

The pectolytic properties of bacteria isolated from the brines of sound and softened cucumbers and olives were investigated. These bacteria, isolated and purified on a solid sodium ammonium pectate medium, were found to include 21 cultures of *B. subtilis* and 22 cultures of *B. pumilus*. All cultures of both species elaborated softening enzymes under suitable conditions.

The softening enzyme complex produced by representative cultures of *B. subtilis*, although active at pH values ranging from approximately 6.0 to 11.0, was most active at pH 8.6. The optimum temperature for its activity was found to be about 45°C.(113°F.) when the reaction time was 30 minutes. The enzyme complex was reasonably stable in alkaline solutions but rapidly lost its activity under acid conditions.

The enzyme complex of *B. pumilus* was quite similar to that of *B. subtilis* except that it was not produced in the beet molasses medium or in any medium tested unless galacturonic acid, pectic acid, or other pectic substances were added to the medium.

Quantitative characterization of the softening enzymes was not successful. However, on the basis of the qualitative experiments performed, it is believed that one component of the softening system is a polygalacturonase-like enzyme similar to that described for *Erwinia aroideae* by Wood *(33)*.

The crude enzyme preparations of both species could soften cucumbers or olives only when the pH of the reaction mixture was at 5.7 or above. It was concluded that the species *B. subtilis* and *B. pumilus* were not involved in the softening of cucumbers or olives which undergo a normal acid fermentation. However, it was pointed out that the bacteria were probably involved in the softening of olives being processed for canned California ripe or green-ripe olives or under certain conditions in spanish-type green olives because of the alkaline conditions created by the use of sodium hydroxide.

Acknowledgments

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FLAVOR DETERIORATION IN FROZEN CAKE BATTERS[a]

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(Manuscript received July 18, 1952)

Sunderlin, Collins, and Acheson *(5)* were among the first to report undesirable flavor development in frozen cake batters. They stated that "cakes baked from the frozen batter were indistinguishable in flavor from freshly baked cakes up to a period of four months. . . . When stored longer than four months the cakes developed an objectionable flavor, probably due to the absorption of locker odors." Somewhat later Graul and Lowe *(1)* reported that off-flavors developed in cake batters containing either hydrogenated lard or butter after 2 months of storage at 0°F.(−18°C.) but not in similar batters stored at −10°F.(−23°C.) up to 6 months. If synthetic vanilla was included in the batters, off-flavors developed during both 0°F.(−18°C.) and −10°F.(−23°C.) storage.

Owen and Van Duyne *(4)* and Zaehringer and Mayfield *(7)*, however, found no flavor impairment in shortened cakes baked from frozen batters up to 4 months of freezer storage. Cakes tested by Owen and Van Duyne *(4)* contained a hydrogenated vegetable oil, while those tested by Zaehringer and Mayfield *(7)* contained butter, lard, or hydrogenated vegetable oil. Neither group tested products stored longer than 4 months. Miller and Beattie *(3)* found no flavor changes in chocolate cakes frozen in the batter state up to 14 weeks of freezer storage.

In a previous publication *(2)* the authors reported the development of off-flavors in sponge cake batters after one month of freezer storage and in shortened cake batters containing hydrogenated vegetable oil after 6 months of freezer storage. It was pointed out that the flavor deterioration was not associated with microbiological decomposition of the batters during freezer storage. Some evidence was presented which indicated that the flavor changes might be due to oxidation of the egg-yolk lipids.

The present study was designed to investigate further the possibility that oxidative deterioration of egg-yolk lipids is primarily responsible for flavor deterioration in cake batters during freezer storage. Peroxide formation was used as an index of oxidation.

PROCEDURE

In the first phase of the study, the concentration of egg yolk and the ratio of egg yolk to total lipid was varied. Six series of sponge cakes and 2 series of shortened cakes were tested. The sponge cakes were of 3 types: egg-white sponge (angel food), containing neither egg yolk nor added fat; whole-egg sponge, containing egg yolk and egg white but no added fat; and egg-yolk sponge, containing neither egg white nor added fat. One series of shortened cakes contained hydrogenated vegetable oil (cottonseed) and the other series contained butter. The cake formulae are shown in Table 1.

[a] Published with the approval of the Vice-Director of the Tennessee Agricultural Experiment Station.

TABLE 1

Cake formulae

Ingredients	Egg-white sponge (angel food) cake	Whole-egg sponge cake	Egg-yolk sponge cake	Shortened cake
Flour (soft-wheat)	156.0 g.	180.0 g.	252.0 g.	284.0 g.
Sugar	387.5 g.	375.0 g.	254.0 g.	340.0 g.
Egg whites	425.0 g.	270.0 g.	.....	60.0 g.
Egg yolks	.....	162.0 g.	252.0 g.	36.0 g.
Salt	2.6 g.	2.2 g.	3.4 g.	8.0 g.
Fat (hydrogenated vegetable oil or butter)	.....	.....	.....	114.0 g.
Water	43.0 ml.	90.0 ml.	214.0 ml.	.....
Milk	.....	.....	.....	285.0 g.
Cream of tartar	4.5 g.	3.9 g.	5.6 g.	.....
Baking powder	.....	.....	12.8 g. (tartrate)	11.4 g. (sulfate-phosphate)
Vanilla (pure extract)	1¾ tsp.	1⅛ tsp.	3¾ tsp.	1½ tsp.

The calculated lipid content of each type of batter was:

	Total lipid content	Egg-yolk lipid content	% of total from egg yolk
	%	%	
Egg-white sponge	0.14	0.00	0
Whole-egg sponge	4.92	4.77	97
Egg yolk sponge	8.32	8.11	97
Shortened cakes	12.27[b]	1.01	8

[b] When butter was used the total lipid content was 10.36%, of which 10% was from egg yolk.

In the second phase of the study the possibility of preventing flavor deterioration in frozen sponge cake batters was investigated. Two series of whole-egg sponge cake batters were frozen in which 30 ml. of lemon juice was substituted for 30 ml. of water to reduce the pH of the batters. The lemon juice constituted 2.8% of the total weight of the batters. Two series of cake batters were also frozen with 0.02% added antioxidant (Griffith G-4WS) containing propyl gallate and citric acid in a corn oil base.

Duplicate tests were made on each type of cake at each storage period. A total of 150 cakes were prepared and tested in the two phases of the study.

Preparation and freezing of cakes and cake batters. All cakes were mixed in a Model K4-B or K5-A KitchenAid electric mixer according to standardized procedures. Details of the preparation of the whole-egg sponge cakes and shortened cakes have been published previously *(2)*. Part of each batch of batter was baked before freezing and part was frozen in the pan in the batter state. Baked cakes and pans of batter were wrapped in cellophane, frozen, and stored in home freezers. The temperatures ranged between 0°F.(−18°C.) and −10°F.(−23°C.) during the period of this study. The sponge cakes were tested at intervals over a period of 6 months and the shortened cakes over a period of 9 months.

Flavor testing. The cakes were graded for flavor by a panel of 5 judges who had preliminary training and experience in grading cakes. The 6 sponge cake samples for a single test period were graded at the same time and in comparison to freshly baked sponge cake. The samples were arranged on a tray in random order and were not identified. Ten points were allowed for odor and 30 points for taste, making the maximum flavor score 40 points. Five quality levels were recognized: *excellent*, *good*, *fair*, *poor*, and *very poor*. The judges assigned to each sample a score corresponding to the estimated quality level. The shortened cakes in the first phase of the study and sponge cakes in the second phase were graded in the same manner but at different times.

Peroxide values. Peroxide values were determined on the lipids extracted from all but the egg-white sponge (angel food) cakes. The cakes were broken into coarse crumbs and air-dried overnight. The crumbs were extracted with chloroform in a Waring Blendor, filtered and the peroxide value of the filtrate was determined according to the procedure of Wheeler *(6)* using 0.01 N sodium thiosulfate.

RESULTS

Flavor changes. Flavor scores of the sponge cakes and shortened cakes tested in the first phase of this study are shown in Table 2.

No flavor deterioration occurred in egg-white sponge (angel food) cakes. There was practically no change in the scores of these cakes during 6 months of freezer storage, whether they were baked before freezing or frozen in the batter state.

The flavor of whole-egg sponge cakes did not deteriorate measurably during 6 months of storage when they were baked before freezing. When frozen in the batter state, however, slight flavor deterioration was detected by some of the judges after only 4 weeks of freezer storage. After 2 months the off-flavor was very pronounced and was observed by all of the judges. It was characterized by a pungent odor and a bitter, metallic taste. Whole-egg sponge cakes frozen in the batter state became inedible between the fourth and sixth month of storage.

Similar off-flavor developed in the egg-yolk sponge cakes which were baked before freezing as well as in those frozen in the batter state. The deterioration was more pronounced in the cakes baked from frozen batters and developed in these cakes at a faster rate than in the baked cakes, or in the whole-egg sponge cakes frozen in the batter state.

No measurable flavor deterioration occurred in any of the shortened cakes which were baked before freezer storage nor in cakes baked from frozen batters containing butter as the shortening. However, 4 of the judges observed slight off-flavor in cakes prepared from frozen batters containing hydrogenated vegetable oil at the 6-month test and definite off-flavor was reported by all judges at the 9-month test. It was similar to the off-flavor developed in the sponge cake batters but much less pronounced.

Peroxide development. Peroxide numbers of the chloroform extract of whole-egg and egg-yolk sponge cakes and shortened cakes are shown in Table 3. There was no noticeable increase in peroxide in any of the cakes which were baked before freezing, nor in the shortened cakes frozen in the batter state during the period of the study. Marked peroxide development occurred in both the whole-egg and egg-yolk sponge cakes frozen in the batter state. After the second month of freezer storage, the peroxide numbers of the yolk sponge cakes increased at a much faster rate than those of the whole-egg cakes.

Batters treated before freezing. The effect of adding lemon juice or a fat anti-oxidant to whole-egg sponge cake batters before freezing is shown in Table 4. The substitution of 2.8% lemon juice for part of the liquid in whole-egg sponge cake batters was effective in preventing peroxide development during the 6 months of testing. Also, it was effective in preventing off-flavor development during 4 months of freezer storage. The addition of G-4WS protected the cake batters from peroxide development for 4 months and against flavor deterioration for 3 months.

TABLE 2

Flavor scores[c] of frozen baked cakes and cakes from frozen batters

Length of freezer storage	Egg-white sponge cakes		Whole-egg sponge cakes		Egg-yolk sponge cakes		Length of freezer storage	Shortened cakes			
	Baked before freezing	Frozen in batter state	Baked before freezing	Frozen in batter state	Baked before freezing	Frozen in batter state		Baked before freezing		Frozen in batter state	
								Butter	H.V.O.[d]	Butter	H.V.O.[d]
None	39	....	40	....	38	....	None	40	39	....	....
2 weeks	39	39	39	36	34	23	2½ weeks	40	39	40	39
1 month	39	39	39	30	33	14	1 month	39	39	39	38
2 months	39	39	38	17	31	8	2 months	40	39	37	37
4 months	38	37	37	15	24	1	6 months	39	39	39	30
6 months	38	37	37	2	11	0	9 months	38	36	39	17

[c] Maximum score, 40. Average of five judges' scores. [d] Hydrogenated vegetable oil.

TABLE 3

Peroxide numbers of the chloroform extract of frozen baked cakes and cakes from frozen batters

Length of freezer storage	Whole-egg sponge cakes		Egg-yolk sponge cakes		Length of freezer storage	Shortened cakes	
	Baked before freezing	Frozen in batter state	Baked before freezing	Frozen in batter state		Baked before freezing	Frozen in batter state
None	3.6	.....	1.9	....	None	2.3	....
2 weeks	3.0	2.9	1.3	2.2	2 months	3.2	2.6
1 month	4.0	5.1	2.1	4.9	4 months	3.2[e]	4.3[e]
2 months	6.1	11.4	3.7	12.3	6 months	3.2	3.5
4 months	5.4	26.3	4.6	36.5	9 months	3.1	2.2
6 months	5.6	45.8	4.9	72.3			

[e] Average of duplicate determinations on one series of cakes. All other values are averages of duplicate determinations on two series of cakes.

TABLE 4

Effects of adding lemon juice or an anti-oxidant to whole-egg sponge cake batters before freezing

Length of freezer storage	Flavor scores[f]			Peroxide numbers		
	Untreated (control)	2.8% lemon juice added	0.02% G-4WS added[g]	Untreated (control)	2.8% lemon juice added	0.02% G-4WS added[g]
None	39	38[h]	39[h]	4.1	4.4[i]	1.1[i]
2 months	14	37	37	9.7	1.9	2.1
3 months	14	38	36	14.1[i]	4.4	1.4
4 months	8	37	31	19.1	1.9	1.8
6 months	0	33	32	56.7	3.2	9.0

[f] Maximum score, 40.
[g] Contains propyl gallate and citric acid in corn oil with added phospholipids.
[h] Average of five judges' scores on one series of cakes. All other figures are averages of five judges' scores on two series of cakes.
[i] Duplicate determinations on one series of cakes. Other figures are averages of duplicate determinations on two series of cakes.

DISCUSSION

There seemed to be a definite relationship between flavor deterioration and peroxide formation in frozen sponge cake batters. Both developments were proportionate to the concentration of egg yolk in the batters. The fact that egg-white sponge (angel food) batters did not deteriorate in flavor during the same test period gives further evidence that egg yolk was probably the source of the off-flavor. In those cakes where flavor deterioration and peroxide formation occurred there was also considerable bleaching of the natural yellow color of the batters, and of the chloroform extract of the cakes baked from these batters, indicating that oxidation of egg-yolk pigments may have been involved also.

The protection afforded by the fat anti-oxidant may also be considered as evidence that an oxidative change (or changes) was involved in the flavor deterioration. The protective effect of the lemon juice is not so readily explained. The pH of the sponge cake batters prepared with water ranged from 5.71 to 6.05; with 2.8% lemon juice the pH ranged from 4.68 to 4.92. The lemon juice may have acted as a direct anti-oxidant; or, if the oxidation was enzymatic in origin, lowering the pH may have rendered the enzymes inactive. If the oxidation was entirely enzymatic, however, no flavor deterioration or peroxide formation should have occurred in the yolk sponge cakes which were baked before freezing.

The nature of the off-flavor which developed in the frozen batters containing hydrogenated vegetable oil cannot be explained on the basis of this work. Up to 9 months of freezer storage, there was no evidence of peroxide development in cakes baked from these batters.

Until more work has been done on the cause and prevention of flavor deterioration in frozen batter products, the freezing of sponge cake batters or other batters high in egg-yolk content cannot be recommended for home or commercial practice. This work would seem to point to the need for long time studies of the performance of frozen batter and dough products as basic to successful marketing of such products.

SUMMARY

That flavor deterioration in frozen sponge cake batters may be caused by oxidation of the egg-yolk lipids during freezer storage is indicated by these findings:

Egg-white sponge cakes did not deteriorate in flavor through 6 months of freezer storage, whether they were baked before freezing or frozen in the batter state.

Whole-egg sponge cakes, with a lipid content of 4.9% of which 97% was from egg yolks, developed off-flavor after 4 weeks and were inedible after 6 months of freezer storage in the batter state. No off-flavor developed when these cakes were baked before freezing.

Egg-yolk sponge cakes with a total lipid content of 8.3%, of which 97% was from egg yolks, developed pronounced off-flavor after 2 weeks and were inedible after 2 months of freezer storage in the batter state. Cakes baked before freezing began to develop off-flavor after 2 months of freezer storage.

There was marked peroxide development in whole-egg and egg-yolk sponge cakes frozen in the batter state. Both flavor deterioration and peroxide formation were proportionate to the concentration of egg yolk in the batters.

The addition of a fat anti-oxidant or lemon juice to whole-egg sponge cake batters delayed the development of off-flavor and the formation of peroxide during freezer storage.

Shortened cakes frozen in the batter state developed slight off-flavor after 9 months of freezer storage when hydrogenated vegetable oil was used, but not when butter was used. No off-flavor developed when shortened cakes were baked before freezing. There was no evidence of peroxide formation in any of the shortened cakes.

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A BACTERIUM RESPONSIBLE FOR A MUSTY ODOR AND TASTE IN ROOT BEER

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The presence of a musty odor, generally accompanied by a similar taste, has, upon occasion, been noted in root beer. It is the purpose of this paper to discuss briefly a species of *Achromobacter* (Aehromobacteriaceae) responsible for this mustiness and to present a differential method for its detection.

Musty odors and tastes have been noted in several foods, and investigators have, in some instances, incriminated species of the genus *Achromobacter* as the causative organism. Turner *(7)* described *Achromobacter perolens* as the bacterium imparting a mustiness to eggs and Spanswick *(6)* corroborated the findings. Musty flavor in butter was attributed by Randell *(5)* to the activities of an unnamed species of *Achromobacter; Achromobacter mucidis* was found by Alford and McCleskey *(1)* to be responsible for a musty crab meat.

PROCEDURES

One-ml. portions of musty root beer were plated with the following media: a, nutrient agar; b, sabouraud agar; c, root beer agar—a media composed of laboratory prepared root beer to which is added 2% agar, and adjusted to a pH of 4.00 by the addition of potassium biphthalate.

The plates were incubated for 96 hours at 29°C. and distinct colonies from nutrient and sabouraud agars were transferred to nutrient broth and thence to their respective agar slants; colonies from root beer agar were transferred to root beer broth and then to root beer agar slants. One-ml. portions of 1 to 100 dilutions of each broth culture (incubated 48 hours at 29°C.) were added to chilled root beer samples, the samples were hand crowned and incubated at 29°C. Control bottles from the same "batch" were inoculated with identical dilutions of the respective, sterile broths. A series of samples and controls were opened after periods of 1, 2, 4, and 8 weeks. One-ml. portions were plated with the 3 previously mentioned media. The beverages were then examined for the presence of musty odors and tastes. Bacteria isolated from experimentally musty beverages were reinoculated into normal root beer. The beverage was then treated as previously explained.

RESULTS

Only the colonies which were detected upon root beer agar were found to be composed of organisms capable of producing a musty odor and taste when inoculated into root beer. The initial "off" characteristics were detected 2 weeks after inoculation and in each subsequently opened sample. Root beer agar pour plates of these samples revealed the presence of typical colonies and reinoculations resulted in musty odors and tastes. All control bottles were normal.

Four different strains, each from a different sample of musty root beer, were chosen for study. A gram stain made from 24-hour cultures of nutrient, sabouraud and root beer agars grown at 29°C., showed plump gram negative rods, growing either singly or in short chains of 3 to 5 individuals;

the width ranged from 0.4 to 0.7 microns and the length from 0.8 to 1.7 microns. Neither capsules nor flagella could be detected by the use of appropriate staining methods (Hiss's copper sulfate capsule stain and Loeffler's flagellar stain).

Colonies on root beer agar, after 96 hours at 29°C., were large, circular to irregular, low convex, transparent, and watery; subsurface colonies were lenticular and white. Nutrient agar surface colonies were tiny, colorless, and convex papillate; subsurface colonies were small, white, and myceloid. Sabouraud agar surface colonies were small, light pink, moist, irregular-rhizoid, and convex papillate; subsurface colonies were myceloid, small, and colorless.

The optimum temperature was not ascertained; growth occurs well at 28-30°C., but not at 55°C., 37°C., room temperature, nor at 5°C. A musty odor is not produced upon any of the media utilized.

The bacterium does not grow in Koser's citrate, produce indol, reduce nitrates, change litmus milk, nor liquefy gelatin; hydrogen sulfide is not produced upon lead acetate agar.

The following fermentation reactions were noted after 3 days: acid, but no gas, was produced in dextrose, maltose, sucrose, and mannitol; in lactose broth 3 strains produced acid but no gas while one strain did not produce acid.

DISCUSSION

The fact that non-sterile samples of root beer were utilized as experimental media might detract from the above noted results, however, the presence of normal control bottles, in all cases, is enlightening, and seems to indicate that this particular species of *Achromobacter* can be responsible for musty flavors and odors in root beer.

The American Bottlers of Carbonated Beverages *Manual of Testing Procedures (2)* and the American Public Health Association *Year Book (3)* prescribes the routine usage of nutrient and either wort or sabouraud agar pour plates for the enumeration of bacteria and yeasts, respectively, in the examination of carbonated beverages. For this reason growth characteristics of the incriminated organism are given upon nutrient and sabouraud agars; however, the cultural peculiarities of the bacterium upon neither of these agars are of sufficient individuality to distinguish the colonies from those of several common, innocuous contaminants; growth upon root beer agar, however, is of great differential validity inasmuch as the species is, to date, the only one encountered, from either normal or musty root beer, which can grow upon the agar in the time and at the temperature utilized in these experiments (96 hours and 29°C.).

Although the organism differs from *Achromobacter perolens* (Turner) and *Achromobacter mucidis* (Alford and McCleskey), and from the 12 recognized species of *Achromobacter* noted in *Bergey's Manual of Determinative Bacteriology* (6th Edition) *(1)*, it is not the writer's intention, at this time, to designate it as a new species. Further work upon the biochemical and physiological attributes of the species, as well as applied research in relation to the carbonated beverage industry, is now in progress and additional communications will be forthcoming.

SUMMARY

An organism of the genus *Achromobacter* has been identified as one capable of causing a musty odor and flavor in root beer. The morphological, cultural, and biochemical attributes of the bacterium have been studied, and the differential value of root beer agar discussed.

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THE METABOLISM OF SORBITAN MONOSTEARATE

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The use of emulsifiers in food products has been greatly increased within recent years. The sorbitan esters of the fatty acids represent one of the newer types which have been found useful as an adjuvant. There is a lack of information regarding their metabolism since these esters do not lend themselves to tracing by specific chemical methods.

The present paper is an investigation of the fate of C^{14} labeled sorbitan monostearate after its oral administration to rats. In some experiments the emulsifier was tagged in the polyol residue and in others, the stearic acid fraction. In this study primary interest has been in the experiments wherein the emulsifier was labeled in the alcohol portion.

METHODS

Animal treatment. Non-fasted male albino rats (190-210 g.) raised on Purina Laboratory Chow were used. The sorbitan monostearate was given orally either as a water emulsion or dissolved in corn oil. The two methods for preparing the test substance for administration were used because the absorption of the emulsifier from the intestinal tract by itself is not necessarily identical with its route when dissolved in oil. Sufficient food and water were supplied the animals for the duration of the experiment. In those experiments in which the expired CO_2 was collected, the animals were placed in individual metabolism chambers *(1)* and the expired CO_2 was then collected for 48 hours in 6-hour intervals. The urine and feces were collected separately as single samples at the termination of the experiment. In Experiments 1 to 14 one rat was used in each.

Animal analyses. At the end of the experimental period the animals were sacrificed for determination of distribution of radioactivity. The liver, kidneys, intestinal tract, and a sample of the hind leg muscle, or the entire carcass, were frozen separately with dry ice and dehydrated by low temperature lyophilization. When the entire carcass was used, the skin was removed in order to facilitate the drying process. The dried tissues were broken into small pieces and extracted with hot $CHCl_3$ in a continuous extractor. The crude fat obtained from the dehydrated skin was included with the carcass fat for radioactive determinations. Parts of the muscle were used in Experiments 1, 2, 8, 9, and 11. In these runs the total radioactivity in the crude fat and non-fat fractions was calculated by assuming that the carcass of the 200-g. rat contained 20 g. of crude fat and 30 g. of non-fat material. These weights approximate the values obtained in the experiments in which the entire carcass was used for analyses. The extraction with $CHCl_3$ served a two-fold purpose. First, the de-fatted tissues are easily disintegrated and pulverized so that uniform planchets could be made for counting purpose. Second, since the emulsifier is soluble in $CHCl_3$, it can be concluded that the radioactivity determined as insoluble in $CHCl_3$ is not in the form of sorbitan monostearate.

For the fractionation of the crude fat, the fat was saponified with hot alcoholic KOH for 6 hours. The alcohol was then evaporated on a steam bath and the residue extracted with petroleum ether to remove steroids. The hydrolysate was acidified and the fatty acids removed by extraction with petroleum ether. The water soluble residue was evaporated to dryness on the steam bath and repeatedly leached with absolute alcohol. The alcoholic extract was evaporated to dryness and the glycerol was removed by sublimation and identified by its index of refraction.

Measurement of C^{14} radioactivity. All radioactive determinations were carried out on $BaCO_3$ obtained directly or by dry combustion, or by direct counting. In all cases the samples were of infinite thickness *(4)*.

Preparation of labeled sorbitol. This was prepared by the reduction of uniformly labeled glucose which had been prepared by plant synthesis *(5)*. The method of preparation and metabolism of sorbitol have been described in a previous publication *(6)*.

Preparation of stearic-1-C^{14}. The acid was prepared by the reduction of heptadecanoic acid to the alcohol with $LiAlH_4$. The alcohol was brominated and the Grignard reagent was carbonated with $C^{14}O_2$.

Sorbitan monostearate. Two g. of sorbitol (sorbitol-carbon[14] for polyol labeled ester) and 4.1 g. of stearic acid (stearic-1-carbon[14] for fatty acid labeled ester) were heated to the melting point in a 3-neck flask and treated with 0.12 ml. NaOH solution (5 g. NaOH in 10 ml. water) and 0.04 ml. 85% phosphoric acid. The mixture was vigorously stirred at 235 ± 3°C. under an atmosphere of carbon dioxide for 45 minutes. The acid, hydroxyl, and saponification values of these products are given in Table 1. It is seen

TABLE 1

Properties of sorbitan monostearate preparations

	Hydroxyl number	Acid number	Saponification number
Sorbitol labeled (Lot No. 1)	230	13	156
Sorbitol labeled (Lot No. 2)	235	11	156
Stearate labeled (Lot No. 1)	240	10	145
Stearate labeled (Lot No. 2)	261	9	145
Sorbitan monostearate[b]	238	10	151

[b] Kindly furnished by Atlas Powder Company, Wilmington, Delaware. Trade mark name is "Span 60."

that our samples are comparable to a commercial sample of sorbitan monostearate (Span 60) with respect to these properties.

Chromatography[a] of polyols obtained by saponification of the commercial emulsifier (Span 60) has shown it to be a mixture consisting of about 70% sorbitan (monoanhydrosorbitol) esters, 16% isosorbide (dianhydrosorbitol) esters and 7% each of sorbitol and mannitol.

RESULTS

Distribution of the radioactivity 48 hours after the oral administration of the sorbitan monostearate is shown in Tables 2, 3, and 4.

Expired CO_2. In all experiments a significant fraction of the administered C^{14} was found in the expired CO_2. The feeding of the polyol labeled ester resulted in recoveries of 14 to 24% of the administered radioactivity. The rate of excretion of C^{14} in the expired CO_2 for some typical experiments is shown in Table 4.

Urine. When the polyol labeled emulsifier was administered in water (Exp. 1 and 2) 25 and 16% of the C^{14} was recovered in the urine. When administered in oil (Exp. 3, 4, 5, and 6) 44 to 66% of the radioactivity was recovered. On the other hand, the feeding of the stearate labeled compound resulted, as expected from the stool analyses, in only a trace of C^{14} excretion.

Stool. The feeding of the stearate labeled emulsifier as a water emulsion (Exp. 7, 8, and 9) resulted in an excretion of 69 to 76% of the fed C^{14} as $CHCl_3$ soluble which is in line with results obtained by absorption

[a] Personal communication from Dr. Peter Kass, Atlas Powder Company, Wilmington, Delaware.

TABLE 2

Percent distribution of radioactivity 48 hours after oral administration of carbon 14 labeled sorbitan monostearate

	Polyol labeled						Stearate labeled				
Experiment No.[c]	1	2	3	4	5	6	7	8	9	10	11
Expired CO_2	15	14	18	19	24	20	18	7	12	33	21
Urine	25	16	66	66	50	44	1	1	1	1	1
Stool											
$CHCl_3$ soluble	31	54	7	6	7	8	75	76	69	37	33
$CHCl_3$ insoluble	17	12	12	8	10	12	3	5	4	2	2
Combined tissues	....	3	6	7	5	7	....	10	15	32	41
Total recovery	88	99	109	106	96	91	97	99	101	105	98

[c] In Experiments 1, 2, 7, 8, and 9 the sorbitan monostearate was emulsified with 8 ml. of water before administration. In Runs 3, 4, 5, 6, 10, and 11 it was dissolved in 4 ml. of corn oil.

Amounts of sorbitan monostearate administered in Experiments 1 to 11 were 240, 1293, 100, 100, 100, 100, 263, 303, 307, 400, and 258 mg., respectively.

The total counts administered in Experiments 1 to 11 were 1.04, 5.6, 2.35, 2.35, 2.35, 2.35, 0.43, 0.49, 0.50, 2.1, and 0.42 million, respectively.

Lot No. 1 (polyol labeled sorbitan monostearate) was used in Experiments 1 and 2, and Lot No. 2 in Experiments 3, 4, 5, and 6. Stearate labeled (Lot No. 1) was used in Experiments 7, 8, 9, and 11, and Lot No. 2 in Experiment 10.

studies on stearic acid *(3)*. When the stearate labeled compound was fed in oil, lower values (33 to 37%) were obtained. Considerably lower results were obtained when feeding the polyol labeled compound. Hence the administration of the esters in oil in all experiments increased their intestinal absorption as indicated by the diminished C^{14} content of the stools.

In order to obtain information regarding the nature of the water soluble urinary material, and the material found in the stool, the excretion products were re-fed to another series of animals. The chloroform soluble extract of the stool from Experiment 2 was administered to a rat (Exp. 12) and the urine from Experiment 2 given to another animal (Exp. 13). The urine from Experiment 12 was also given to a rat (Exp. 14). The distribution of the radioactivity 48 hours after the feeding is shown in Table 5.

TABLE 3

Percent distribution of radioactivity in tissues 48 hours after oral administration of C^{14} labeled sorbitan monostearate

	Polyol labeled					Stearate labeled			
Experiment No.[d]	2	3	4	5	6	8	9	10	11
Liver									
$CHCl_3$ soluble	0.06	0.08	0.09	0.10	0.11	1.38	1.45	4.30	4.70
$CHCl_3$ insoluble	0.23	0.52	0.55	0.57	0.50	0.35	0.62	0.92	1.82
Kidneys									
$CHCl_3$ soluble	0.01	0.01	0.02	0.08	0.03	0.18	0.05	0.14	0.18
$CHCl_3$ insoluble	0.02	0.05	0.05	0.05	0.05	0.05	0.14	0.09	0.49
Intestines									
$CHCl_3$ soluble	0.07	0.29	1.18	0.12	0.76	0.61	0.62	2.53	2.54
$CHCl_3$ insoluble	0.18	0.98	0.77	0.59	1.64	0.18	0.23	0.73	0.85
Carcass									
$CHCl_3$ soluble	0.53	1.13	1.39	0.68	0.91	6.20	11.30	17.95	26.40
$CHCl_3$ insoluble	1.44	2.58	2.85	2.84	2.80	1.45	1.07	5.65	3.90
Total recovery	2.54	5.64	6.90	5.03	6.80	10.40	15.48	32.31	40.88

[d] The experimental runs are the same as Table 2.

TABLE 4

Rate of C^{14} excretion in expired CO_2 after administration of polyol labeled sorbitan monostearate [a]

Period (hours)	Experiment number			
	3	4	4	6
1- 6	3.2	2.1	3.2	1.8
7-12	4.9	4.2	4.9	2.3
13-18	4.8	3.8	4.1	2.6
19-24	2.7	4.6	4.2	1.8
25-30	1.1	2.5	2.5	4.5
31-36	0.7	1.0	1.9	4.2
37-42	0.6	0.7	2.1	1.4
43-48	0.3	0.4	1.0	1.4
Total	18	19	24	20

[a] The experimental runs are the same as Table 2.
Values expressed as % of fed ester.

TABLE 5

Percent distribution of radioactivity 48 hours after administration of urine and stool extracts

	Stool [f] from No. 2	Urine from No. 2	Urine from No. 12
Experiment No.	12	13	14
Expired CO_2	28	23	24
Urine	27	64	34
Stool			
$CHCl_3$ soluble	32	5	5
$CHCl_3$ insoluble	17	24	31
Total	104	116	94

[f] Chloroform soluble material.

Body residual radioactivity. A small amount (3-7%) of the ingested radioactivity was recovered in the tissues 48 hours after the feeding of the polyol labeled compound. Under similar conditions a 4- to 6-fold increase in C^{14} deposition occurred by feeding the stearate labeled compound when dissolved in oil.

The distribution of the C^{14} in the crude fat obtained from animals given 100 mg. of sorbitan monostearate (Lot 2) dissolved in 4 ml. of corn oil is shown in Table 6. Three animals were sacrificed after 2 and 7 days, respectively. In these experiments the carcasses included the kid-

TABLE 6

Distribution of C^{14} in crude carcass fat [g]

Time of sacrifice after feeding	2-day	7-day
1. Fatty acids	38	42
2. Glycerol	27	43
3. Residue [h]	14	12
Total [i]	79	97

[g] Values expressed as percent of the crude fat.
[h] Residue is the water soluble material which did not sublime.
[i] Values for steroid fraction in all cases amounted to less than 0.2% of the crude carcass fat.

neys and the liver. Although the crude fat obtained from each animal was saponified separately for the isolation of the fatty acids, the water soluble fractions were combined for the 2- and 7-day animals, respectively. By averaging the results from the 3 animals in each of the 2- and 7-day groups, the percentage of the injected C^{14} in the crude fat (excluding the intestinal tract) was 0.76 and 0.46%.

DISCUSSION

It is apparent from the data obtained that approximately 90% of the sorbitan monostearate, when administered to rats in oil solution, is hydrolyzed either in the intestinal tract, or after its absorption, or both, to stearic acid and the anhydrides of sorbitol. The urinary material containing C^{14} (44 to 66% of the fed C^{14} in Exp. 3-6) is believed to represent the anhydrides which were liberated by the splitting of the ester. These anhydrides of sorbitol are excreted by the kidneys to a large extent before they can be completely oxidized to CO_2. The fact that they can be oxidized was clearly demonstrated when they were re-fed to other animals. It would appear that complete oxidation would take place if the kidneys were removed so as to prevent excretion. It has been assumed *(2)* that the respective anhydrides of the sugar alcohols are not metabolized in the animal body, which is understandable in the light of our findings concerning the rapid excretion of these substances. The re-feeding experiments also indicate that the anhydrides are poorly absorbed from the intestinal tract. These results are not surprising since it has been previously shown *(6)* that the intestinal absorption of sorbitol was slower than that of glucose. The relatively constant amount of C^{14} in the expired CO_2 in all experiments is further evidence that the sorbitol anhydrides are oxidized by the animal.

The ester was fed (Exp. 3, 4, 5, and 6) at the high level of 0.5 g. per kilo weight of rat. This corresponds to 30 g. of a 60-kilo man. The high level feeding was necessary in order to obtain sufficient radioactivity for counting after its dilution in the body. Under these abnormal conditions only a relatively small amount of C^{14} was found in the tissues as chloroform soluble. The incorporation of C^{14} in all of the tissue constituents was inevitable in the presence of labeled CO_2 and other tagged intermediates. Fractionation of the carcass fat showed, as expected, that C^{14} was incorporated into the fatty acids, glycerol, and non-sublimable residue fraction. This residue may represent, in part, polyols derived from the fed ester. It can be calculated that the "residue" represents only 0.10 and 0.05% of injected C^{14} (equivalent to 0.1 and 0.05 mg. of the fed ester) in the 2- and 7-day groups, respectively.

SUMMARY

Radioactive sorbitan monostearate has been fed to rats and the fate of the C^{14} has been studied.

The distribution of the C^{14} shows that at least 90% of the polyol labeled emulsifier, when fed in oil solution, was hydrolyzed to stearic acid and the anhydrides of sorbitol. When fed as a water emulsion, about 50% of the ester is hydrolyzed.

The anhydrides of sorbitol which were liberated by the hydrolysis were largely excreted into the urine before they could be completely oxidized to CO_2.

The amount of C^{14} found in the tissues 48 hours after feeding of polyol labeled sorbitan monostearate in oil was 5 to 7% of the administered C^{14}. Fractionation of the crude fat extract of the tissues (excluding the intestinal tract) indicated that less than 0.1% of the fed C^{14} may represent sorbitans derived from fed sorbitan monostearate or sorbitan esters synthesized from circulating sorbitan.

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THERMAL RESISTANCE OF LACTIC ACID BACTERIA AND YEAST IN ORANGE JUICE AND CONCENTRATE[a]

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(Manuscript received June 11, 1952)

Frozen concentrated orange juice as it is generally produced is not heated and, therefore, is not bacteriologically sterile. It is essential that strict and efficient bacteriological control be maintained constantly over the manufacture and canning operations to insure a low total count and minimum contamination with microorganisms capable of growth and production of off flavor in the product. Murdock *et al. (2)* observed certain cultures of lactic acid bacteria to produce off flavor in single strength juice, 20° Brix concentrate and 42° Brix concentrate. Yeasts, occurring as a large percentage of the microflora normally present, also may cause spoilage. Several instances of off-flavor spoilage of commercially packed frozen concentrate have occurred as the result of growth of these organisms.

Heat treatment of juice prior to evaporation or of the concentrate to inactivate the pectic enzymes is the most commonly accepted method to prevent gelation and clarification *(6)*. Some packers are seriously considering heat treatment of the juice or concentrate to eliminate this difficulty. There appears to be no published data on the thermal resistance of the yeasts or lactic acid organisms in single strength or concentrated orange juice. Since the pasteurization of the product at some stage during manufacture might accomplish both the objectives of preventing gelation and clarification, and the elimination of organisms producing off flavor, data on the thermal resistance of these organisms in juice and concentrate are of interest and value in suggesting possible pasteurization procedures.

EXPERIMENTAL PROCEDURE

Three composite stock suspensions were prepared. One composite consisted of 11 strains isolated from orange concentrate and tentatively identified as *Leuconostoc* based on morphology, catalase reaction, fermentation of sucrose and pentoses, and gum forming properties. Included also in this composite were one strain each of *Leuconostoc mesenteroides*.[c] The second composite consisted of two strains of *Lactobacillus brevis* and one strain of *Lactobacillus fermenti*.[d] The third composite consisted of 13 strains of yeasts isolated in this laboratory from single strength juice or concentrate.

All of the test organisms were grown on Orange Serum Agar slants for 48-72 hours at 30°C. Each slant was washed with 5 ml. of sterile water and the suspensions combined in sterile bottles with glass beads to give the respective composites. The suspensions were standardized by plating on Orange Serum Agar pH 5.4 and counting the plates after 48 hours of incubation at 30°C. The standardized suspensions were stored in the refrigerator when not in use. Thermal resistance was determined in single strength juice (9.8° Brix, pH 3.7, total acidity 0.91%) and concentrate (42° Brix, pH 3.45, total acidity 3.62%).

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[a] Presented at the Twelfth Annual Meeting of the IFT, Grand Rapids, Michigan, June 11, 1952.

[b] Presently associated with Minute Maid Corp., Plymouth Florida.

[c] Received from Dr. Carl S. Pederson and Dr. Reese H. Vaughn.

[d] Received from Dr. Carl S. Pederson.

TABLE 1

Corrected survival and destruction times for Leuconostoc, lactobacilli and yeasts

Stock composite	Organisms per ml.	Single strength orange juice											
		130°F.		135°F.		140°F.		145°F.		150°F.		155°F.	
		Surv. min.	Dest. min.	Surv. min.	Dest. min.	Surv. min.	Dest. min.	Surv. min.	Dest. min.	Surv. min.	Dest. min.	Surv. min.	Dest. min.
Leuconostoc	340,000	24.1	34.1	4.2	6.2	0.8	1.1	....	....	....	....	....	....
Lactobacilli	1,000,000	....	....	39.1	....	6.1	8.1	1.1	2.1	....	....	....	....
Yeasts	860,000	....	....	....	....	17.1	24.1	....	2.1	0.3	0.4	....	....
		Orange concentrate (42° Brix)											
Leuconostoc	130,000	18.9	28.9	4.8	6.8	....	....	0.7	1.2	....	....	....	....
Lactobacilli	275,000	11.9	16.9	....	....	3.7	6.7	....	....	0.7	1.7	....	....
Yeasts	600,000	....	....	....	....	....	....	23.7	33.7	5.7	8.7	1.2	1.7

Juice or concentrate in bottles with glass beads was inoculated with a known quantity of a composite suspension and after thorough shaking, 2 ml. of the inoculated product was distributed into sterile cotton-stoppered thermal death time tubes (9 × 120 mm.) which were then sealed with an oxygen torch. The tubes were exposed in a constant temperature bath at different temperatures for various periods of time, removed and rapidly cooled. Five replicates were used for each time period at each temperature. The contents of the tubes were transferred to sterile plates which were then poured with 15-20 ml. of Orange Serum Agar pH 5.4. The plates were incubated 5 days at 30°C. and examined for the presence or absence of growth. The destruction point was taken as the shortest period of time showing no growth on any of the replicate plates, the survival time was the next shorter period of exposure, or the longest period showing any survival on the replicate plates. The total exposure times were corrected for heating lag following the procedure of Sognefest and Benjamin *(4)*.

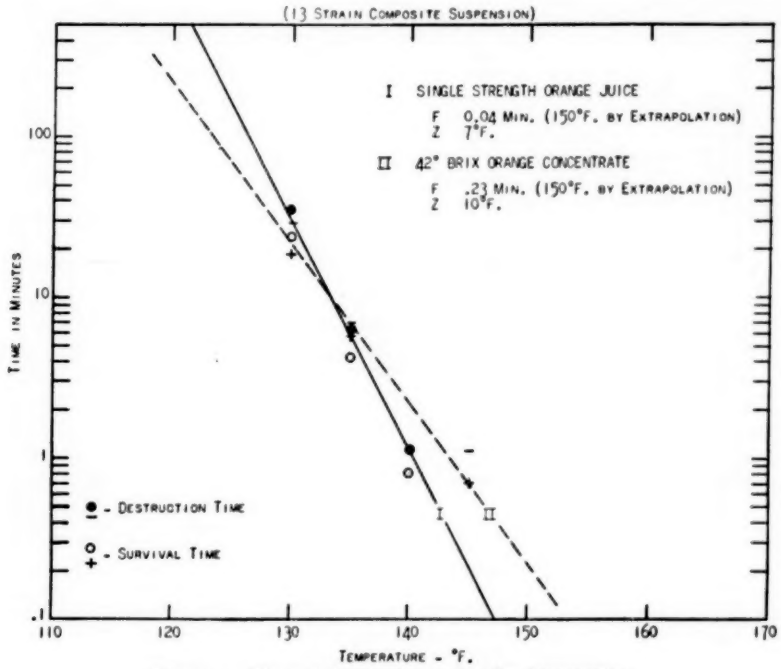

Figure 1. Thermal death time curve for leuconostoc.

RESULTS

The survival and destruction time data are presented in Table 1. Thermal death time curves obtained by plotting this data on semi-logarithmic paper are shown in Figures 1, 2, and 3. Townsend, Esty, and Baselt *(5)* discuss the development of thermal death time curves and the use of F and z to characterize the thermal resistance of a suspension. A reference temperature of 150°F. has been used in this work. Table 2 shows a summary of the F and z values and the ratio of resistance in concentrate to resistance in juice for these organisms.

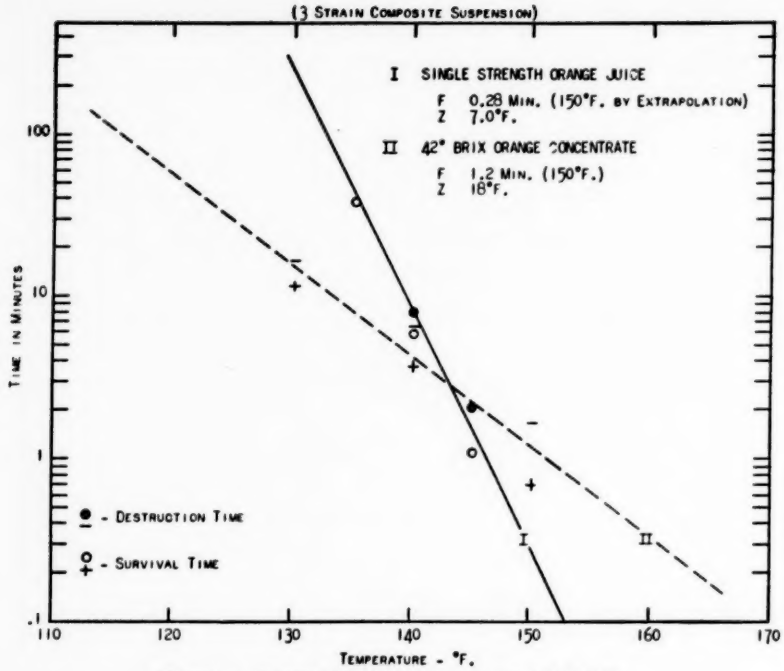

Figure 2. Thermal death time curve for lactobacilli.

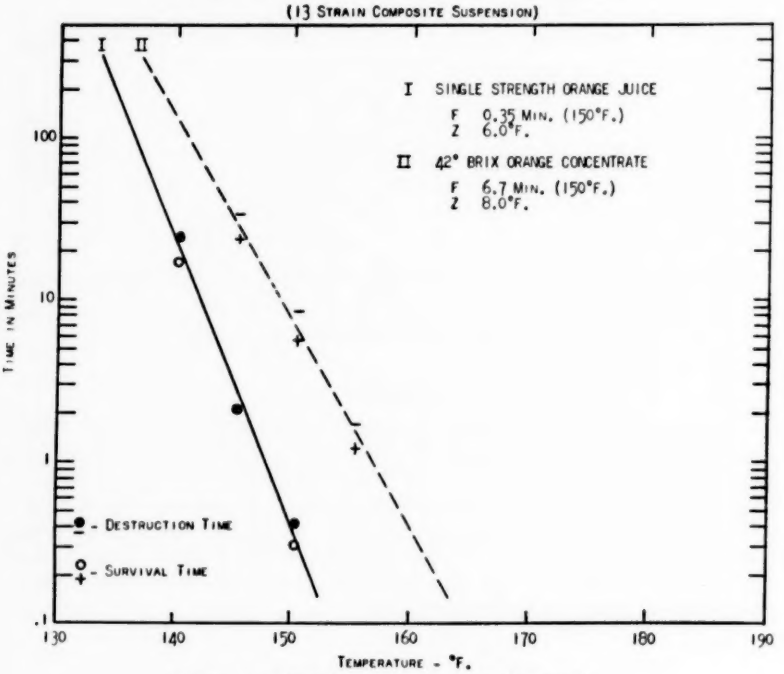

Figure 3. Thermal death time curve for yeasts.

DISCUSSION

The yeasts exhibited a greater resistance in both single strength juice and concentrate, and also showed the greatest increase in resistance in concentrate as compared to juice. Peterson *et al. (3)* found that yeasts were more rapidly destroyed at 212°F.(100°C.) in distilled water than in syrup. Anderson *et al. (1)* observed that increased concentrations of sucrose or dextrose in tomato juice enhanced the thermal resistance of *Bacillus thermoacidurans*. These results would suggest the desirability of applying the pasteurization process to single strength juice rather than to concentrate as a greater lethal effect may be expected for any given process, or less process will be required to obtain a given degree of lethality.

TABLE 2

Summary of thermal resistance of test organisms in single strength orange juice and 42° brix concentrate

Test organism	Single strength juice		Concentrate		Ratio of resistance in concentrate to resistance in juice
	F_{180}	Z	F_{180}	Z	
Leuconostoc	0.04	7	0.23	10	6
Lactobacilli	0.28	7	1.2	18	4
Yeast	0.35	6	6.7	8	19

SUMMARY

The thermal resistance of 3 composite suspensions, consisting respectively of 13 strains of *Leuconostoc*, 3 strains of lactobacilli, and 13 strains of yeast, has been determined in single strength orange juice and 42° Brix orange concentrate. The organisms were found to have greater resistance in concentrate than in single strength juice, possibly due to the increased sugar content. These results suggest that any proposed commercial pasteurization of this product may be more effectively applied to the single strength juice than to the concentrate.

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